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FURTHER OBSERVATIONS ON THE DEMONSTRATION OF AN INFECTIVE AGENT IN THE TISSUES OF FOWLS AFFECTED WITH FOWL PARALYSIS (NEUROLYMPHOMATOSIS)

By

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INTRODUCTION

Earlier studies showed that a virus could be demonstrated in the tissues of fowls suffering from fowl paralysis (Blakemore, 1939, 1941). It was also found that serial passage of the infective agent through highly susceptible in-bred chicks increased its virulence, a fatal disease then frequently occurring within a few days. The survivors of this acute disease were unthrifty and in a large proportion lesions of neurolymphomatosis later developed. Resistance to the infection increased with age, it being impossible to stimulate visible lesions in adults. Glover (1940) confirmed the virus nature of fowl paralysis with material from a turkey and he passaged the infecting agent through 22 groups of chicks. In Glover's experiments, however, the late lymphomatous process appeared less often in survivors of the acute disease. Asplin (1944), working with field cases of neurolymphomatosis, has also demonstrated a virus which produces a similar effect in young chicks and becomes much more virulent after passage at short intervals.

Further observations on the virus recovered from outbreaks of fowl paralysis are now reported and an account will be given of (*a*) the disease produced by the inoculation of field material in young chicks, (*b*) the susceptibility of different stocks, and (*c*) the chief properties of the causal agent.

Stock of Fowls used in the Experiments.—The transmission work was carried out mostly with the P strain of fowls used in earlier work (Blakemore, 1939). This stock, derived originally from a flock infected with fowl paralysis, has now been closely in-bred for nine generations. The incidence of the disease was high in the first two generations but subsequently the progeny have been free of it. The successive generations of fowls were killed off after each had been observed for a period extending from one to two years, the examination to which they were then subjected including histological study of the nerves and viscera. Although after rearing in isolation the disease has not occurred amongst them, these in-bred fowls have proved highly susceptible to it on exposure to natural infection or on inoculation with material from affected fowls.

THE RECOVERY OF VIRUS FROM FIELD CASES OF FOWL PARALYSIS

To confirm the earlier findings that lesions can be set up in young chicks by the inoculation of material from cases of neuro-lymphomatosis, the following experiments were conducted. In the first series eleven outbreaks were examined.

A 1 per cent. suspension of the lymphomatous lesions of the killed donors was prepared by triturating with sand in a sterile mortar. After removal of the coarse matter by slow centrifugation 0·5 c.c. was inoculated into one to three day-old chicks intraperitoneally, each inoculated group comprising 6 to 14 chicks. They were kept under observation for varying periods, strict isolation being maintained between the units. During the experiments precautions were taken to exclude nutritional deficiencies and intercurrent infection. Control P chicks of the same generation were under observation at the same time.

The chicks were killed at varying intervals after inoculation, the viscera examined for lesions, and sections prepared of the principal organs. As indicated in Fig. 1, the development of lesions could be demonstrated in all except two of the groups but in some chicks they were microscopic. The majority of the chicks showed no symptoms during life, and although the most severe lesions were usually observed in sick chicks this was not invariably so. The disease produced with material from one source (V) was transmitted to a second group, which resulted in an increase in its severity, many of the chicks dying within a short time.

In a further series of experiments, using fowls affected with neurolymphomatosis from three further sources, the infective agent was maintained by serial passage through P chicks and the results were briefly as follows.

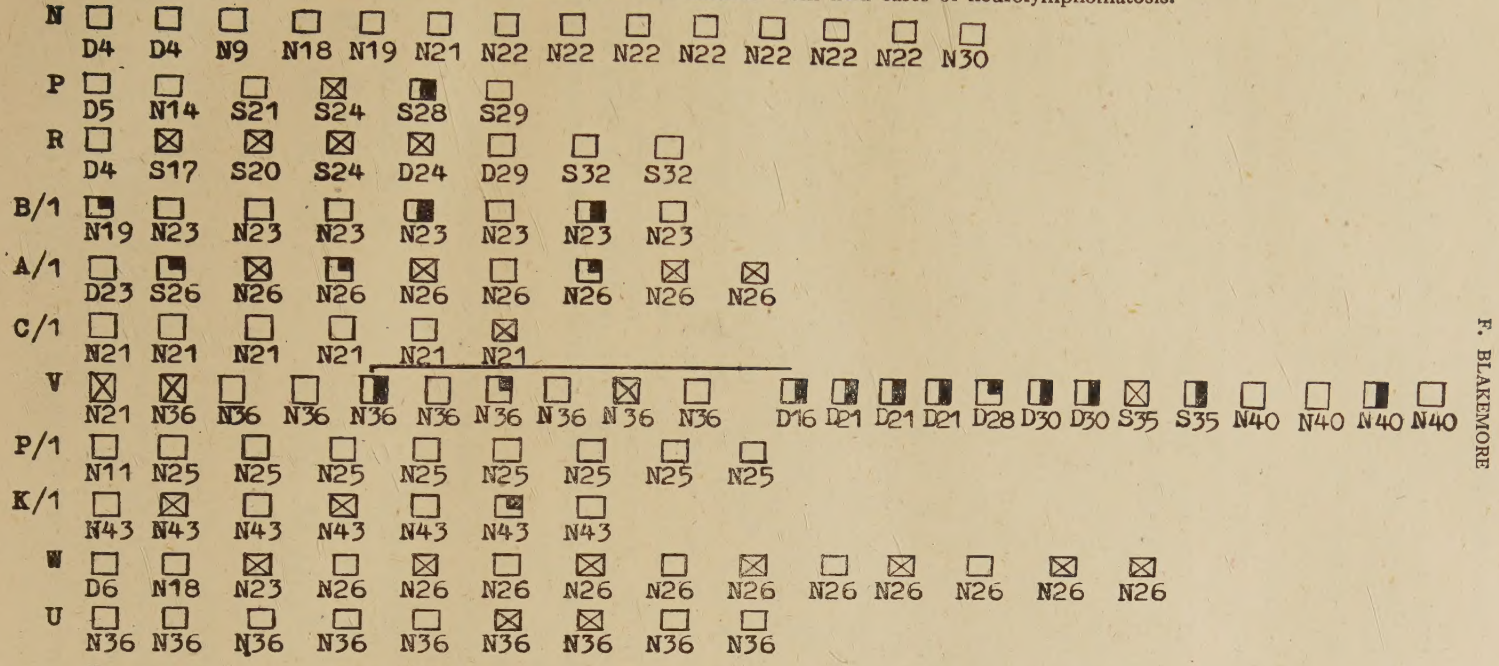
Virus B.—The infective material was obtained from a pullet flock with a history of paralysis preceded by unthriftiness. Examinations revealed nerve lesions, lymphomata, and diffuse infiltrations of the viscera. Some of the first chicks inoculated were sick in 14 days and the average time for the development of lesions was 25 days. Material from the first chick to be examined was serially transmitted to two further groups and the severity of the disease increased, all the chicks in the third passage group dying within ten days. These results are represented in Fig. 2, which will also be referred to later in connection with the effect of the virus on three-month-old fowls.

Virus X.—This was derived from a small flock in which there were occasional losses from fowl paralysis, the actual donor showing enlarged nerves and lymphomatous liver lesions. The infection became established on inoculation without difficulty and it was subsequently passaged through ten groups of chicks. This resulted in an increase in virulence as was made evident by the fact that the average killing time at the eighth passage was reduced to only six days.

Virus L.—Information was lacking regarding the outbreak but the fowl selected was affected with neural and visceral lymphomatosis. Again, no difficulty was encountered in demonstrating the presence of an infective agent, which was passaged through six groups. As in previous experiments,

FIG. 1.

Groups of chickens inoculated with material from field cases of neurolymphomatosis.



Explanation of Signs (Figures 1, 2 and 3). □ chicks. ○ 3 month old fowls.
Lesions □ (○) no significant abnormality. ■ (●) Gross nerve lesions.
Visceral lesions:- ◪ slight. ◨ marked. ⊠ microscopic.
Numerals indicate days after inoculation when examined.
S = sick. D = died. N = no symptoms. P = paralysed.

the virulence of the virus was increased considerably, for although only mild symptoms were observed after an average of 21 days in the first group, the chicks in the third died within four days.

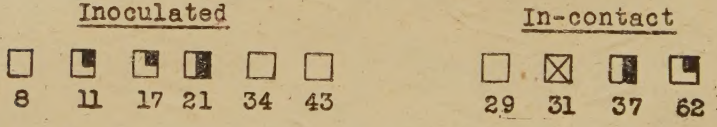
The disease produced in all these experiments was essentially of the same character but differed in severity. In the groups receiving field material symptoms, if they appeared at all, were mild, the chicks showing slight unthriftiness and isolating themselves from their fellows. There were also certain changes in the plumage, the feathers of the wings and thighs growing to an abnormal length. The appetite, however, was good and the few which were retained improved considerably.

With material of high virulence obtained after rapid passage, the majority of inoculated chicks died in a few days without showing any definite symptoms. Those which lived for 7 to 14 days appeared listless and were disinclined to move away from the brooder and there was slight diarrhoea. The feathers were ruffled, their wings were relaxed, and they trembled excessively when exposed to cold.

While intraperitoneal inoculation was adopted as the standard method of infection, transmission was also successful by the intravenous, subcutaneous and intramuscular routes. When the last-named route was followed no local reaction tended to arise. The disease was also found to spread by contact, indicating the need for maintaining different experimental units in strict isolation. Indeed, strains of virus were occasionally propagated by mixing susceptible chicks with affected groups and usually in such cases there was a period of 20 to 40 days between exposure and the development of lesions. An example of a typical in-contact experiment is depicted, in Fig. 3. In this case normal chicks were mixed with the infected chicks ten days after they had been inoculated and there was an interval of 31 days before the first proved case of the disease occurred in the exposed group.

FIG. 3.

Illustrating the spread of infection to in-contact chicks.



For explanation of signs see Fig. 1.

The possibility was borne in mind that the results obtained in these experiments might have been due to a subclinical infection in the P stock which was activated by the injection of fowl tissue and increased in severity by passage. That this was not so was shown by an experiment in which the tissues of day-old healthy P chicks were pooled and inoculated into others of the same batch.

The inoculated chicks developed normally and no lesions were found in them when they were killed after 20 to 40 days. Furthermore, their tissues were inoculated into another group with the same negative result.

VARIATION IN SUSCEPTIBILITY OF DIFFERENT STOCKS OF CHICKS TO THE VIRUS AND ITS EFFECT ON OLDER FOWLS

Although the P stock was principally used for transmission work, the virus has been determined to be also infective for other stocks, but during the investigation it became apparent that there are varying degrees of resistance between different breeds and even individuals. One stock of chicks was found to be almost totally refractory and the initial success in establishing the infection experimentally was no doubt largely attributable to the use of highly susceptible chicks. Many of the failures to reproduce lymphomatosis may possibly have been due to the use for transmission purposes of stocks in which the natural incidence of the disease was low, since with a universally prevalent disease these probably represented resistant strains. To confirm this view chicks of four stocks reported to be resistant to fowl paralysis and a batch of P chicks were inoculated simultaneously when all were 48 hours old with a constant dose of virus. All the P chicks died before the 12th day but the majority of the chicks of different origin developed normally and most of them showed no gross lesions at 25 days (Table I).

TABLE I

VARIATION IN SUSCEPTIBILITY TO THE ACUTE DISEASE AMONG DIFFERENT CHICK STOCKS

48-hour-old Chicks	*No.*	*Mortality*			*Survivors 25 days*	*Survivors affected*	*Total incidence*
		24 hrs.	*24 to 72 hrs.*	*72 hrs. to 18 days*			
P stock	4	0	2	2	—	—	4/4
Stock 1	12	1	2	1	8	1	5/12
„ 2	12	0	4	0	8	0	4/12
„ 3	12	0	1	0	11	2	3/12
„ 4	12	1	1	0	10	1	3/12

Field evidence of an inherent variation in susceptibility to fowl paralysis among different stocks of fowls has been adduced and commented on by a number of workers. Biely *et al.* (1933) found a similar tendency in fowls which were artificially infected by the inoculation of lymphomatous tissue. They judged the incidence of the disease by the development of classical lesions of neurolymphomatosis and recorded that of the groups inoculated four families comprising 27 fowls remained unaffected, in eight families there were

49 normal and 42 paralysed fowls and in a further five families the infection rate was 16 of the 78 fowls employed. A similar result was obtained by Dalling and Warrack (1936) since in their transmission experiments in which two strains were employed they found one to be more susceptible than the other. They also concluded that lymphomatosis was a condition associated with individual birds and recorded a high incidence in the progeny of certain fowls. Basing his assumption principally on information of this kind, Oakley (1935) advanced the view that lymphomatosis was in the nature of an hereditary neoplasm. Because of the association between a virus and lymphomatosis it could be argued that the field disease was the effect of chronic infection in susceptible stocks of birds. It is quite possible, however, that other factors are concerned, and these must be considered. When birds belonging to the P stock became affected with the acute disease many of the survivors developed lymphomatosis. It must be stressed, however, that only a limited number of observations have been made on other stocks but some evidence was obtained that stocks of chicks that are resistant to the virus show only a low incidence of neurolymphomatosis in later life. In one experiment carried out to throw further light on this point, 19 chicks belonging to a relatively resistant stock were inoculated intraperitoneally with a virus of high virulence, eleven being kept under observation for 159 days. In those examined shortly after infection there were hardly any visceral changes, and only one of the eleven maintained for the duration of the experiment showed nerve lesions and these were microscopic.

It was found in a previous investigation that chicks of the P stock became resistant to the virus with advancing age and the results are summarised in Table II. The majority of the 19 fowls which were 8 to 20 weeks old at the time of inoculation became paralysed, and when killed massive areas of infiltration, predominantly lymphocytic, were found in the nerves, heart and liver. In the nine aged 18 months to two years, however, the organs were either normal or showed only healed lesions and there was no involvement of the nerves. Three of the virus strains referred to (B, X and L), after repeated passage, were inoculated into three-month-old P fowls, and the results which are included in Table II, were somewhat similar six developing lesions. Taking all these results into consideration, the average time for the development of neural lesions was 93 days for 8-week, 88 days for 12-week and 119 days for 20-week-old fowls. With one of the viruses (L) the nerves of only one of the five inoculated fowls became affected, but it cannot be concluded that it had less affinity than the other strains for nervous tissue since the numbers in each group were small. The rise of virulence of virus B after its recovery from the tissues of a naturally infected fowl and the effect subsequently produced on older birds are represented in Fig. 2.

TABLE II

THE EFFECT OF THE VIRUS ON FOWLS OF DIFFERENT AGES

	Experiment	Age at inoculation	No. of fowls employed	Period between inoculation and killing	Neural lesions	Gross visceral lesions	Microscopical visceral lesions	Total incidence
				Days				
Original virus	1	8 weeks	4	30 to 225	4	4	0	4/4
		20 „	5	44 to 281	4	4	1	5/5
		18 months	5	43 to 296	0	0	1	1/5
		2 years	4	43 to 281	0	0	1	1/4
	2	12 weeks	5	38 to 105	5	5	0	5/5
	3	20 „	5	149 to 168	4	4	1	5/5
Virus X	4	12 „	4	75 to 262	2	2	0	2/4
L		12 „	5	237 to 279	1	0	0	1/5
B		12 „	5	79 to 262	3	3	0	3/5

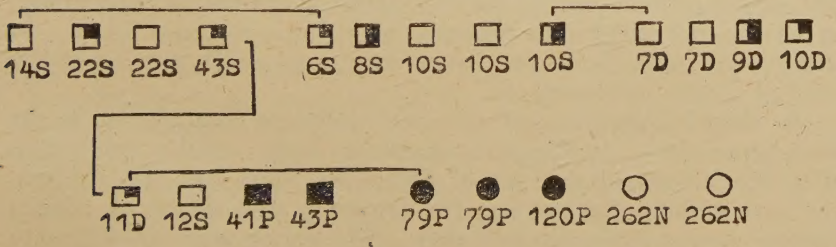
The development of an age immunity to the virus, in the sense of an increased resistance of fowls with advancing age which cannot be attributed to previous infection, is a matter of special interest. It is evident that there is a period during the life of the fowl when the lymphomatous reaction is more likely to become apparent. Indeed, resistance would seem to develop slowly with age, and the tendency for lymphomata to be produced is apparently associated with a partial immunity of the tissues.

PATHOLOGY

The diagnosis of the acute disease was based on the presence of gross or microscopical lesions in the viscera and the exclusion of bacterial infection. This procedure was of limited value, however, and failed to detect all infected chicks. For example, in the case of virus B (Fig. 2) the first subject in the initial passage did not show

FIG. 2.

Indicating the rise in virulence for chickens of virus B and its effect after passage on three-months old fowls.



For explanation of signs see Fig. 1.

lesions although it was sick. It was infected, however, as evidenced by the demonstration of virus in its tissues. The pathological studies made in this investigation were mainly concerned with the prelymphomatous stage of the disease in chicks and reference is made to the chronic condition (neurolymphomatosis) only to indicate the relationship of the two diseases in the experimental stock.

Chicks which died a few days after inoculation often showed no distinct lesions but occasionally in them the pericardial sac was distended with clear serous fluid and areas of necrosis with haemorrhage were apparent in the liver. The identification of the disease then depended on histological examination and the exclusion of infection. In cases of longer duration the musculature was slightly oedematous and lesions which were essentially similar were found in the heart, liver and occasionally the other organs. The liver was slightly enlarged, its capsule was sometimes covered with a gelatinous exudate and throughout its substance there were ill-defined greyish white areas. The heart was also increased in size and while in some there was a serofibrinous pericarditis with adhesions, in others the changes were limited to the myocardium where there was localised or diffuse infiltration. In many cases these infiltrations persisted for many weeks until the age when neural lesions could be expected, but as the disease became chronic they tended to localise and become more truly lymphomatous, often appearing as neoplasms of diverse sizes. The diffuse type of infiltration affecting the myocardium and the development of lymphomata are depicted in Fig. 4. It was sometimes, however, difficult to classify the lesions on this basis since at all stages gradations of reaction were encountered.

Histological examinations were made at different times after inoculation in order to trace the development of the disease.

The tissues were fixed in 10 per cent. formol saline and haematoxylin and eosin were employed as the routine stains although on occasion van Gieson and a modification of Foot's silver impregnation method were used.

In severely affected chicks which died a few days after infection, sections revealed areas of necrosis, which were especially prominent in the liver, involving the cells in the vicinity of the small vessels and often the vascular structures themselves, without any cellular reaction in the surrounding tissues (Fig. 5). In some there were haemorrhagic areas in the liver and the appearance resembled, but was not so pronounced, as that described by Duran-Reynals (1940) in chicks inoculated intravenously with Rous sarcoma virus. In these acute cases there was serous exudation which was especially marked in the pericardial sac.

In older lesions (10 to 40 days after infection) the reaction in the liver assumed a proliferative character, with cellular infiltration (Fig. 6). Localised necrosis of the parenchyma was also evident, but one striking feature was the tendency for islands of normal tissue to persist in the diseased areas. The vessels were still affected; in some there was irregular growth of the endothelium into the

lumina occasionally leading to occlusion. In others there were outgrowths of fibrillary tissue from the vessel walls, as was rendered evident by the demonstration of argyrophil fibres (Figs. 7 and 8), which in many instances appeared to grow unrestricted and surround groups of normal cells, thus suggesting a neoplastic tendency. The picture was often complicated by lymphocytic infiltration giving the appearance of lymphoblastoma and frequently there was fibroblastic activity. The fibrillary network was also marked in the heart and not infrequently there were outgrowths into the pericardial cavity. In more advanced cases these proliferative changes were not so regularly demonstrated and only localised fibrotic thickenings of the vessels and portal tracts remained.

The cellular reaction which was present from the tenth day onwards appeared to arise principally from the haemopoietic foci (Fig. 9) which are related to the vessels of the liver and other tissues. In many instances it was almost entirely lymphocytic but in some of the lesions there were also extensive accumulations of polymorphonuclears, myelocytes and histiocytes, the latter often being plentiful around the lymphocytes where they appeared to form a limiting zone. Centrally placed giant cells were occasionally seen.

In chicks of resistant stocks the lesions were similar to those encountered in the P stock, but they were not well marked and tended to regress in 20 to 30 days. In many of the P chicks, especially in those inoculated with field material, there was a similar tendency but in others the acute type of response with proliferation of fibrillary tissue, fibroblastic activity and mixed-cell infiltrations persisted for a long time. In some cases, however, the lesions became frankly lymphomatous, increasing in size until they were later found as tumour-like masses in the heart, liver and occasionally the other organs.

While the changes in the experimental pre-lymphomatous phase differ in certain respects from those found in cases of field fowl paralysis, there are obviously points of resemblance. An examination of sections of the organs from cases of neurolymphomatosis clearly indicates that the conception commonly held of the disease, *i.e.*, of progressive lesions gradually increasing in size and consisting solely of lymphocytes produced locally from lymphoblasts, is incorrect. That this is so is indicated by the early study of the pathology of the condition by Pappenheimer *et al.* (1929), in which they indicated the variety of the tissue response and observed necrotic and granulomatous lesions in the myocardium very similar to those described in the experimental chicks. Moreover, they drew attention to the evidence of transition between lesions which appeared inflammatory and those which assumed the character of a true neoplasm. Vascular involvement of the viscera was described by Potel (1938), who mentioned the occurrence of subendothelial collections of cells arranged in half loops or diffusely, and he also referred to activation

of the capillary endothelium in the nerves. The findings of this investigation support Potel's observation. Indeed, it is not uncommon to find vascular thickenings and endothelial proliferations unassociated with the gross lesions in organs containing lymphomata.

The fact that the liver and heart were principally involved in chicks is of interest since it is these organs in which lesions are commonly found in acute bacterial infections, *e.g.*, *S. pullorum* disease. The liver is perhaps the organ most commonly seen to be affected in field neurolymphomatosis and the heart is not infrequently affected, being specially commented on by Jungherr (1944), who also mentioned that there is sometimes a localised pericarditis. A similar observation was made by Shiwmeister (1939), and he too described degenerative changes in the myocardium.

While certain of the early changes described in the experimental chicks are indicative of a necrotic inflammatory process, others suggest neoplasia. This latter tendency cannot be excluded on account of the pleomorphism of the response or the retrogression of lesions, since these features have been observed during the early stages in the development of fowl tumours, *e.g.*, they were described by McIntosh (1933) in the case of tar-induced avian neoplasms. The transitory nature of the general disturbance, which from the parts affected would suggest a stimulation of tissue of mesoblastic origin and the tendency for the lesions to regress or to become frankly lymphomatous is, however, in conflict with the view that a multiplicity of specific conditions finally follows the inoculation of a single virus—a view which has been advanced by a number of workers.

Neural changes were not expected in chicks used in these experiments as these changes take some time to develop, but perivascular infiltrations and necrosis of the midbrain were occasionally seen in a few which developed complete paralysis.

One experiment was carried out with the object of ascertaining whether the virus had any special affinity for nervous tissue. A suspension of infective material was inoculated by the intracerebral route into a young P cockerel and serially transmitted by the same route at intervals of 14 days to six others, using nervous tissue on each occasion. There was no evidence that this procedure produced lesions in the nervous system or affected the virus since the typical acute disease was produced in chicks with brain suspensions from the last cockerel used. An experiment of this kind does not give sufficient evidence to warrant the conclusion that the affinity of the virus for nervous tissue cannot be increased or exclude altogether the possibility of it possessing a neurotropic action. While the lesions in the peripheral nerves in cases of neurolymphomatosis cannot be fully accounted for, it does seem probable, however, that they are principally due to a stimulation of the vessels and interfibrillary structures followed by lymphocytic infiltration between the fibres.

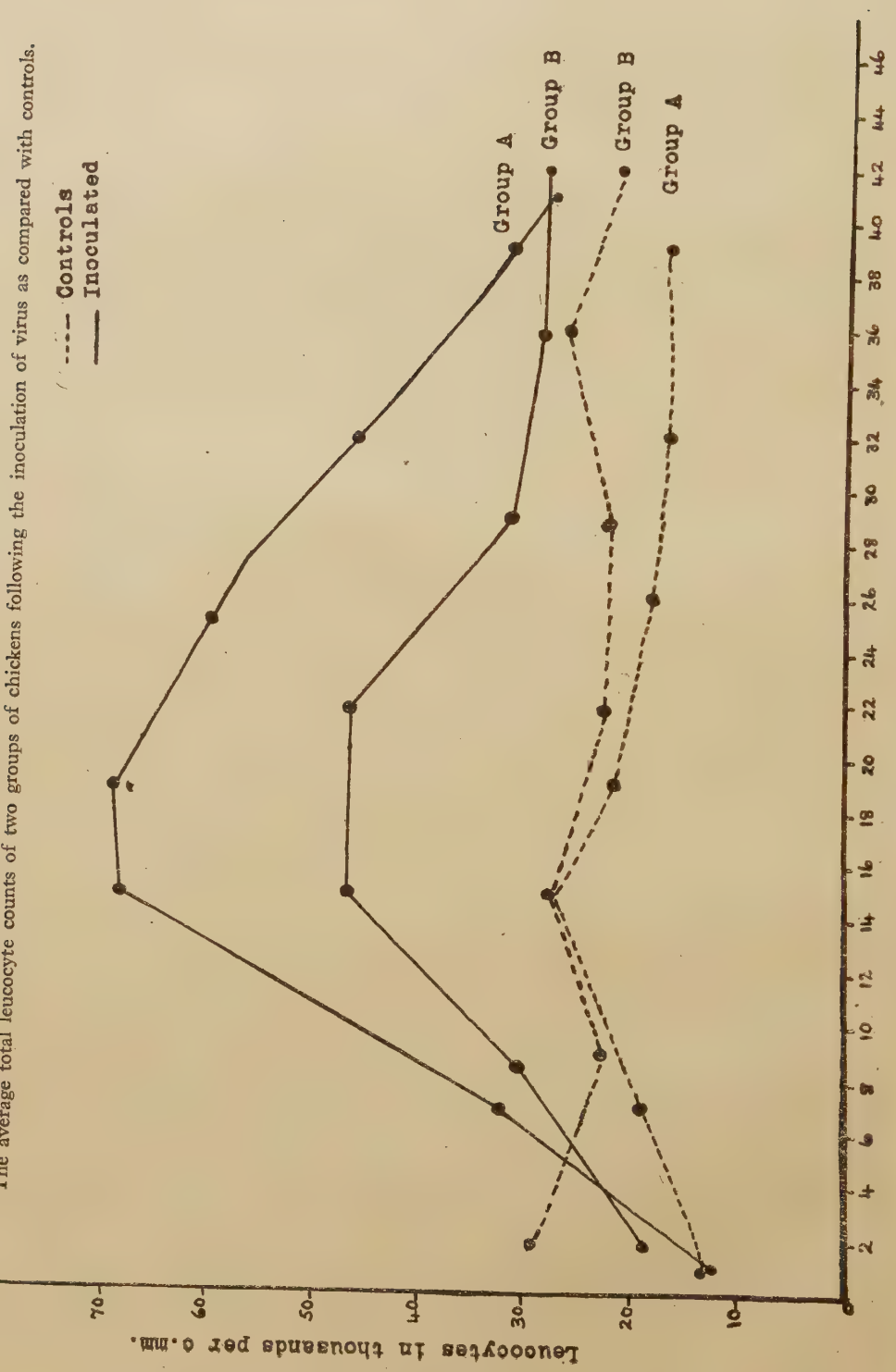
RELATIONSHIP TO LEUKAEMIA

There has been a tendency to classify neurolymphomatosis as a leukaemic disease and to regard this disease, erythroleucosis, and myeloid leucosis as due to the same virus or closely related viruses. The relationship of the last two mentioned conditions was established by Stubbs and Furth (1931), but the observations of Hall and his co-workers (1941) would tend to support the view that neurolymphomatosis is also caused by the same aetiological agent. Working in America with inbred stock, Hall demonstrated the presence of an infective agent, believed to be filtrable, in material from two cases of neurolymphomatosis which was maintained by serial passage through young stock and would infect embryos. Some of the inoculated fowls developed erythroleucosis and others lymphomatosis but the percentage showing the former type of response tended to increase with passage of the virus and the opinion was formed that it became of a true leucosis type.

In earlier experiments (Blakemore, 1939) erythroleucosis was not observed, and after regular passage of this original virus in P chicks for five years, lymphomatosis still occurred amongst the survivors in some of the inoculated groups but others showed only the prelymphomatous type of lesion. Insufficient evidence has yet been obtained by long-term experimentation on which to form an opinion as to whether the ability of the virus to produce lymphomatosis is decreasing, but there are indications that this may be so. The nature of the acute lesions in chicks inoculated with the passaged virus shows no change, however, and there is no evidence that it is now able to produce other pathological processes. In view of the results obtained by Hall, it was decided to examine the blood of chicks infected with the virus after it had been passaged repeatedly for five years to ascertain whether there was then any indication of mutation as evidenced by its ability to produce leukaemia.

Two experiments were carried out using four seven-day-old chicks in each group with an equal number of controls and the total leucocyte counts were determined at intervals (Fig. 10) over a period of 42 days. A rapid rise was evident during the early stages of the disease but while the increase was considerable 10 to 28 days after inoculation, by the 40th day the counts were only slightly higher than in the controls and not significantly above the values sometimes obtained in healthy fowls. These results do not indicate any leukaemic tendency in the experimental condition and it is interesting to note that one of the chickens in Group A became paralysed seven days after the experiment was terminated. The erythrocyte counts remained throughout within the normal range and only a few erythroblasts were observed. The haemoglobin index of representative specimens, determined at intervals of seven days by the alkali haematin method, was constant.

Differential counts were made on Group A over a period of 39 days and the findings are given in Table III. The determinations



The average total leucocyte counts of two groups of chickens following the inoculation of virus as compared with controls.

were carried out on films stained by Leishman and for the purpose of investigation the large and small lymphocytes were classified together as were the basophil, eosinophil, and pseudoeosinophil polymorphs. The primitive cells which were only numerous when the highest counts recorded were included in the series to which they belonged, although in some cases identification was difficult.

TABLE III

AVERAGE LEUCOCYTE COUNTS OF INFECTED AND CONTROL CHICKS (GROUP A) FOLLOWING THE INFECTION OF VIRUS

Days after infection	*Infected*				*Controls*			
	Lymphocytes	*Polymorphs*	*Monocytes*	*Total leucocyte counts*	*Lymphocytes*	*Polymorphs*	*Monocytes*	*Total leucocyte counts*
1	60% 7,574	30% 3,787	10% 1,262	12,624	75% 9,937	10% 1,325	15% 1,987	13,250
7	42% 14,418	43% 14,761	15% 5,149	34,330	62% 12,028	32% 6,208	6% 1,164	19,400
14	50% 34,275	36% 24,678	14% 9,597	68,550	61% 16,958	36% 10,008	3% 834	26,410
17	43% 29,583	35% 24,055	22% 15,126	68,730	60% 13,260	39% 8,619	1% 221	22,100
32	50% 20,932	33% 13,815	17% 7,117	41,865	71% 12,638	23% 4,094	6% 1,068	17,800
39	55% 15,180	31% 8,556	14% 3,864	27,600	74% 12,876	21% 3,654	5% 870	17,400

It was found that the leucocytosis was primarily due to an increase in cells of the lymphocyte and monocyte series. There was also a slight rise in the polymorphs, including pseudopolymorphs, but myelocytes were rarely seen and there was no basophilia. The increase in the number of monocytes is of interest as this has been a prominent feature persisting for a considerable time in some of the other groups which have been examined.

In Hall's experiments the leucosis was associated with marked enlargement of the spleen and liver and it was found that whereas in the normal chicks averaging 196 g., the percentage weight of the spleen was 0·26 and the liver 3·9, the value in affected specimens was 1·7 and 10·6, respectively. In the present investigation the relative weights of these organs in relation to the body weight was determined in groups of six chickens of various ages which had been inoculated when one to three days old with the passaged virus referred to in the preceding experiment. This procedure was considered desirable in view of the fact that the proportional weights

of the organs may alter with advancing age and a marked abnormality was not expected. The results given in Table IV indicate some increase in the weight of the organs, especially in chickens below 160 to 180 g., but there was no appreciable enlargement in the more chronic disease. This result, therefore, enhances further the general findings, supporting the impression gained of an acute but transitory tissue disturbance during the early stages of the disease and differentiates between the condition and that described by Hall.

TABLE IV

PERCENTAGE WEIGHTS OF THE ORGANS IN RELATION TO BODY WEIGHT

Body weight in grms.	*Affected*		*Normal*	
	Spleen	*Liver*	*Spleen*	*Liver*
20 – 30	0·89	6·5	0·02	3·6
40 – 50			0·18	4·4
50 – 60			0·11	3·7
60 – 70	0·19	6·5	0·09	3·2
70 – 80	0·33	5·7	0·08	2·9
80 – 90	0·25	6·0		
90 – 100				
100 – 120	0·50	5·3		
120 – 140	0·40	5·4	0·10	3·8
140 – 160	0·30	4·5	0·18	4·3
160 – 180	0·45	3·2	0·15	3·6
180 – 200	0·34	5·5	0·11	3·4
200 – 250	0·15	4·7	0·80	3·0
250 – 300	0·16	3·6	0·10	3·0

THE INFECTIVE AGENT

All attempts to cultivate a micro-organism from chicks infected with the acute disease have failed and inclusion bodies have not been demonstrated in the tissues.

For filtration and other experiments it was found necessary to use virus of high virulence obtained by the rapid passage of material through young chicks. By this means it was possible to obtain suspensions which were infective at dilutions of 10^{-6}. The virus was found to pass through tested Berkefeld V filters and Gradacol membranes of 0·6 μ porosity, but consistently negative results were obtained with Berkefeld N filters, indicating that the virus is comparatively large.

The most satisfactory way of maintaining the virus was found to be by stimulating a chronic response in two to three-month-old P fowls and by adopting this procedure it could readily be recovered several months after inoculation. Though it quickly disappeared from tissue even when it was kept in the refrigerator, fully virulent suspensions have remained viable for seven days and buffered liver

suspensions containing 50 per cent. glycerine were infective after they had been kept for five days. The effect of heat was tested by heating suspensions to 56°, 60° and 70° C. for 30 minutes and it was found that the lowest temperature was sufficient to destroy the virus completely.

Two experiments were conducted using sera from paralysed fowls several months after inoculation to ascertain whether any inactivating substance was present. Virus dilutions varying from 10^{-1} to 10^{-6} were mixed with equal quantities of neat serum, and after standing for two hours the mixtures were inoculated intraperitoneally, using six chicks in each group. There was some evidence of neutralisation as judged by the severity of the lesions when the chicks were killed after 14 days, but adult normal serum inactivated the virus to almost the same limit. This result is in agreement with the findings of Glover (1940), who also failed to confer passive immunity with sera from chronically infected fowls and it would seem that circulating antibodies are not produced to any marked extent.

Attempts to adsorb the virus on to red cells were successful. For this purpose an equal amount of packed fowls' red cells was added to a 1 per cent. suspension of chronically affected liver. After one and a half and 18 hours in the ice chest the mixture was centrifuged, the cells removed, resuspended in the same amount of saline and some groups of chicks inoculated with suspension and others with supernatant. The chicks were kept under observation for seven days and the presence of virus was based on the development of gross lesions. As indicated in Table V, there was evidence of partial adsorption at one and a half hours and it was complete at 18 hours. Subsequently, the experiment was repeated with equine red cells and the findings were similar. It was also found that the virus remained attached to the cells after two successive washings.

TABLE V

ADSORPTION OF VIRUS ON ERYTHROCYTES

Material	*Time*	*No. of chicks*	*Numbers affected*		
			1–7 days	*Survivors affected at 7 days*	*Total*
	Hours				
Fowl cells ...	1½	4	0	2	2/4
Supernatant ...	1½	4	0	1	1/4
Fowl cells ...	18	4	1	3	4/4
Supernatant ...	18	4	0	0	0/4
Equine cells ...	18	8	4	3	7/8
Supernatant ...	18	8	2	1	3/8

DISCUSSION

The results obtained confirm earlier findings that a virus can be demonstrated in cases of neurolymphomatosis by the inoculation of tissue suspensions into chicks. The virus can be increased in virulence by rapid passage and the character of the disease changed from an almost symptomless condition to one in which death often occurs within a few days. To demonstrate the virus in this way it is necessary to employ highly susceptible chicks since some stocks are comparatively resistant and the virus has little effect upon them. The real significance of this inherent resistance to the disease is a subject for further study, but it is obviously of practical importance and may explain why in the field certain breeds, stocks and individuals are more severely affected than others.

The tendency for a number of different conditions to occur in fowls inoculated with material from cases of fowl paralysis has led to the belief that it and the leukaemic conditions are due to a single virus. In this study of the experimental disease in young chicks it was evident that the changes leading to lymphomatosis were of a varied nature but even after repeated passage the virus showed no marked alteration. Moreover, there was no tendency for it to produce other conditions such as endotheliomata and myelomatosis as was experienced by Furth (1933) or erythroleucosis as reported by Hall *et al.* (1941). Because of the prevalence of leukaemia and allied diseases, many of which are loosely defined, the results of some of these experiments reported in the literature in which a multiplicity of conditions is reported following the inoculation of material from field cases of neurolymphomatosis, may be explained by differences in interpretation or by dual infection, but the position can only be clarified by long-term experiments carried out with closely inbred stock maintained in strict isolation.

In lymphomatosis there is an early stimulation of tissues of mesoblastic origin and the lesions appear to arise principally as a result of damage to the vascular system with secondary activation of those haemopoietic centres which are intimately associated with the small capillaries. In the embryo these foci are concerned with blood production (Nonidez, 1920), but their function during maturity is not fully understood although they are obviously activated in leukaemia. When lymphomatosis develops the effect produced is similar to leukaemia in that these centres are stimulated to unrestricted activity, but lymphomatosis differs from true leukaemia in that the process is usually localised to certain organs or even nerves and the principal blood producing centres are not affected. It is generally agreed that the two diseases are closely related but it may be dangerous to draw too close an analogy or apply too strictly the principles of mammalian pathology to birds.

The nature of neurolymphomatosis is a point of considerable interest, especially in view of the transition from a necrotic inflammatory process to one which is so proliferative that the lesions may

appear neoplastic on autopsy. Lymphomatous reactions are known to occur in some chronic infections for massive deposits enveloping egg material which has escaped from the oviduct following rupture, have been observed, and Downham (1939) described lymphomata around the liver lesions in blackhead. Furthermore, the marked cellular reaction observed in cases of tuberculosis, which is often accompanied by an increase in numbers of leucocytes sometimes to a level of 200,000 per c.mm., led to the early belief that this disease was really responsible for the lesions which Ellermann attributed to leukaemia (Engelbreth-Holm, 1942). On the other hand, it may be pointed out that the lesions produced in resistant stocks inoculated with Rous sarcoma may resolve before they reach the neoplastic stage; in addition, a marked histological pleomorphism from mesoblastic stimulation occurs during the development of tar-induced avian tumours (McIntosh, 1933). Moreover, the haemorrhagic response produced by Duran-Reynals (1940) in young chicks and embryos with Rous sarcoma virus, which was attributed to necrosis of cells of the vascular system, is a feature of the acute disease. He also obtained evidence that the neoplasia caused by chicken tumour viruses of high activity evolves only when the host is endowed with a certain resistance, due to the appearance of an age-developing, virus-suppressing, antibody. It is in these respects that fowl-paralysis virus resembles the filtrable tumour agents.

In the case of fowl paralysis the lymphomatous reaction is associated with the persistence of virus and the lesions would seem to represent a chronic response, but it cannot be assumed that this observation applies to all susceptible strains. Indeed, it may be that the tissues of fowls behave differently under the influence of virus stimulation as the result of inherent intrinsic factors such as have been described in mouse leukaemia. This possibility, as well as the existence of a multiplicity of related viruses, must be taken into consideration when attempting to account for the development of a variety of different diseases attributed to a single agent, especially as in the literature more than one condition is often described in a single group of inoculated fowls.

From the practical point of view neurolymphomatosis should obviously be regarded as contagious and likely to spread by contact amongst chicks during early life. The development of non-susceptible stocks would appear to be perhaps the most important method of limiting losses, but a policy of accepting freedom from the disease as evidence of resistance is not without danger, since the incidence in the stock used in these experiments was invariably high when they were exposed to infection, whereas they remained healthy when in isolation.

It remains to be ascertained whether a condition comparable to the acute experimental disease is responsible for chick losses on affected farms and investigations into this aspect of the problem are being made. The disease has already been identified in the field, but it seems likely that fowl paralysis usually develops as a symptomless condition causing only a limited mortality during the first weeks of life.

SUMMARY

The infectious nature of fowl paralysis was confirmed by the inoculation of suspensions of lymphomatous lesions into susceptible chicks. Fourteen outbreaks were examined in this way and a virus was demonstrated in 12. In the first group of chicks to be inoculated the response was mild but by serial passage it was possible to increase the virulence of the virus.

A marked variation was found in the susceptibility of chicks of different origin to the early response which followed the inoculation of virus and a resistant stock showed only a low incidence of neurolymphomatosis. The view that this latter condition represents a chronic stage of the experimental disease was supported by the demonstration of an age-developing, virus-suppressing state and a higher incidence of neurolymphomatosis in fowls of a susceptible strain inoculated before this became fully developed.

The infective agent was found to be filtrable and behaved like a virus. There was no evidence that the virus stimulated a multiplicity of specific neoplasms or leukaemic conditions and even after repeated passage it showed no tendency to become of a true leukaemic type.

ACKNOWLEDGMENTS

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REFERENCES

Asplin, F. D. (1944). *Nature*, **153**, 253.
Biely, J., Palmer, V. E., and Lloyd, E. A. (1933). *Proc. 5th World's Poultry Congress, Rome.* 143.
Blakemore, F. (1939). *J. comp. Path.*, **52**, 144 : (1941). *Proc. roy. Soc. Med.*, **34**, 109.
Dalling, T., and Warrack, G. H. (1936). *Vet. J.*, **92**, 310.
Downham, K. D. (1939). *Ibid.*, **95**, 89.
Duran-Reynals, F. (1940). *Yale J. biol. Med.*, **13**, 217.
Engelbreth-Holm, J. (1942). *Leukaemia in Animals*, London.
Furth, J. (1933). *J. exp. Med.*, **58**, 253.
Glover, R. E. (1940). *Vet. J.*, **96**, 427.
Hall, W. J., Bean, C. W., and Pollard, M. (1941). *Amer. J. vet. Res.*, **2**, 272.
Jungherr, E. (1944). *Diseases of Poultry*, Biely and Biester : Iowa.
McIntosh, J. (1933). *Brit. J. exp. Path.*, **14**, 422.
Milford, J. J., and Duran-Reynals, F. (1943). *Cancer Res.*, **3**, 578.
Nonidez, J. E. (1920). *Amer. J. Anat.*, **28**, 81.
Oakley, C. L. (1935). *Proc. roy. Soc. Med.*, **28**, 999.
Pappenheimer, A. M., Dunn, L. C., and Cone, V. (1929). *J. exp. Med.*, **49**, 63.
Potel, K. (1938). *Ztsch. f. Infektionskr.*, **54**, 43.
Shiwmeister, E. (1939). *Archiv. f. Geflugelk.*, **9**, 300.
Stubbs, E. L., and Furth, J. (1931). *J. exp. Med.*, **53**, 269.

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FIG. 4. The upper photograph illustrates the diffuse type of response in the heart of a chick 15 days after experimental infection. Note the greatly increased size of the heart. The figure below shows the development of well defined lymphomatous growths in the heart and liver five months after infection. Only a portion of the liver is shown. The size of the heart is approximately normal. (The photographs are natural size.)

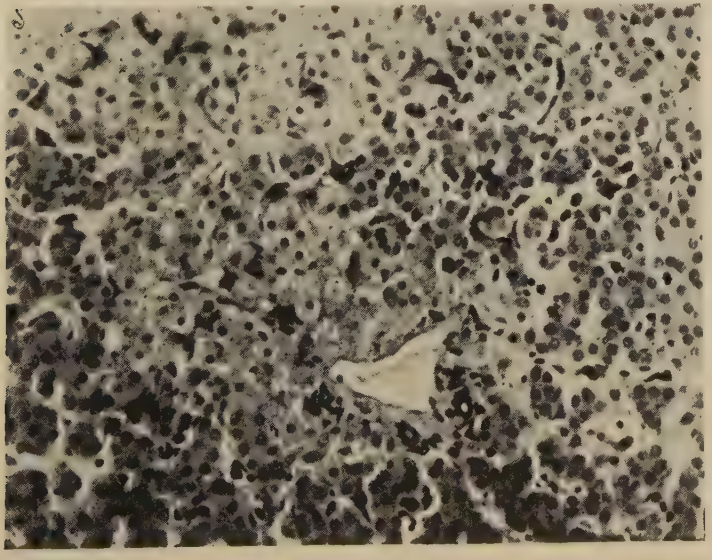

FIG. 5. Liver seven days after the inoculation of virulent virus, showing perivascular necrosis. Haematoxylin and eosin. ×300.

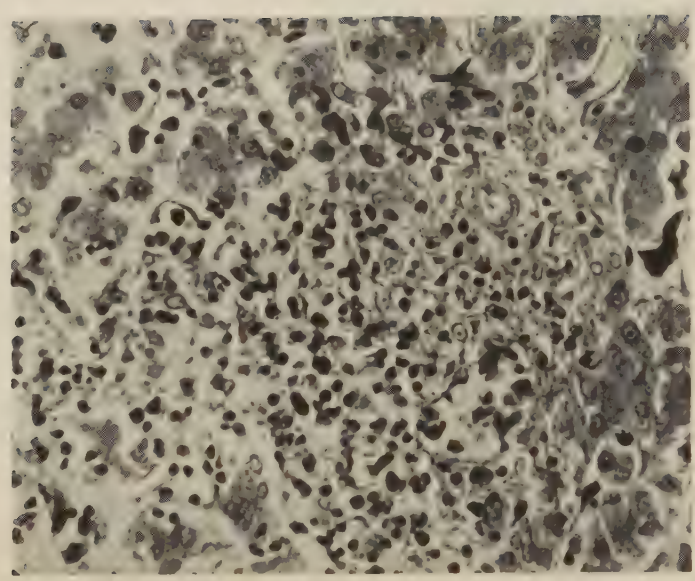

FIG. 6. Liver 15 days after infection. Note the early lymphocytic infiltration and necrosis. Haematoxylin and eosin. ×400.

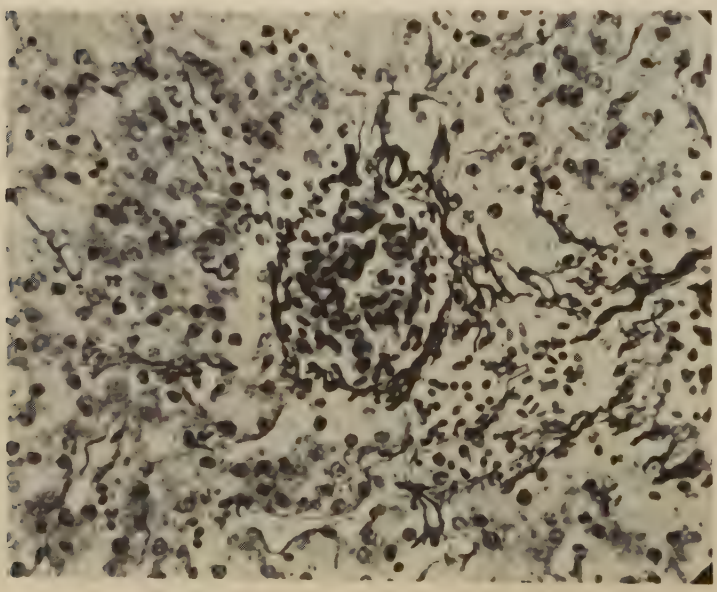

FIG. 7. Liver showing some perivascular necrosis and the proliferation of silver stained fibres from the vessel ten days after infection. Modified Foot's silver impregnation method. ×400.

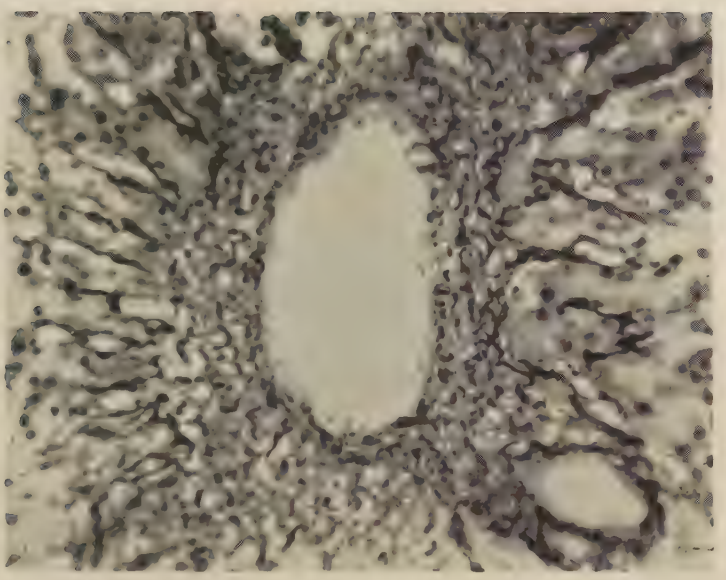

FIG. 8. Liver 24 days after infection. There is thickening of the vessel wall and considerable proliferation of silver stained fibres. Modified Foot's silver impregnation method. ×400.

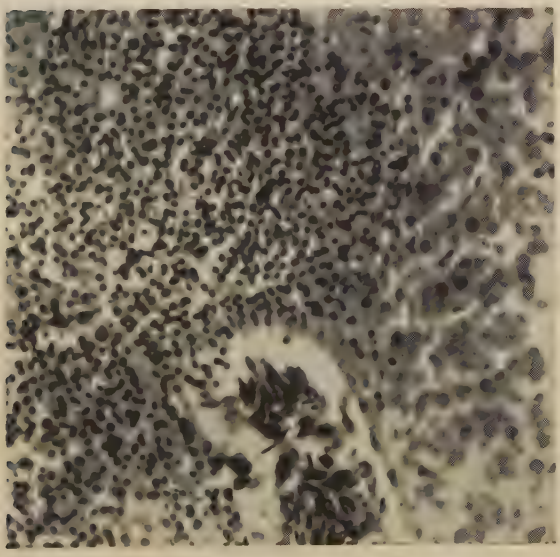

FIG. 9. Liver showing stimulation of perivascular haemopoietic centre 25 days after infection.
Haematoxylin and eosin. ×250.

CONTRIBUTIONS TO THE STUDY OF SWAYBACK IN LAMBS

I. FIELD EXPERIMENTS

By

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INTRODUCTION

Following the work of Bennetts and Chapman (1937) on enzootic ataxia or swayback of lambs in Australia, Dunlop and Wells (1938) in this country confirmed the previous findings that feeding Cu supplements to the pregnant ewe greatly reduced the incidence of swayback in the ensuing lamb crop. Repetition of the work a year later by Dunlop, Innes, Shearer and Wells (1939), with concomitant pathological and biochemical studies, showed that the use of salt licks containing 0·3 per cent. Cu was very effective in preventing the disease, and that ewes in the swayback area of North Derbyshire had a very low blood Cu status. Innes and Shearer (1940) published a comprehensive account of the disease and its pathology, and Shearer, Innes and MacDougall (1940) extended these observations. The general practical recommendation from all this work was to feed salt licks containing copper sulphate, as a prophylactic measure to ewes in affected areas.

A survey of the disease position in hill sheep in the North Derbyshire area some two years later showed that swayback (or " warfa " as it is known locally) was still the greatest single cause of losses in lambs despite the widespread use of copper licks. On some farms the evidence suggested that copper was of little practical value. Licks were placed in troughs protected from the weather but there was no guarantee that they were regularly consumed by all the sheep. It was therefore decided to re-open the whole question of the prophylactic value of copper and of other trace elements by more individual methods on selected farms, and at the same time carry out a systematic investigation of the copper status of each animal. By having a small number of farms under more complete control we have had a better opportunity of making detailed studies than was afforded to the previous investigators in this area.

Another factor in the alleged failure of copper to offer protection against swayback was the fact that some of the licks sold commercially in the area contained amounts of copper sulphate less than that previously recommended. Consequent failures created a lack of confidence by farmers in the area in the value of licks in general, and led to half-hearted or intermittant adoption of prophylaxis.

After arrangements with regard to compensation for lambs affected with swayback, five farmers agreed to place their flocks at disposal. All had histories of heavy losses from the disease in

previous years and were prepared to co-operate on an experimental basis, involving division into groups and dosing at regular intervals. Another group of 18 farmers, who could not co-operate as fully as was desired, agreed to use commercial licks and keep full records of consumption and of their losses from swayback. A third experiment was arranged for 40 ewes to be purchased from farms of known swayback history, and transferred to the laboratory for attempted experimental induction and control of the disease by careful control of diet. The experiments thus comprised three parts, which may now be separately reported.

I. DERBYSHIRE: MAIN EXPERIMENTAL GROUP ("DOSE" FARMS)

The ewes of this group, distributed over five farms, totalled 371 initially. On each farm at the end of October the sheep were divided into four sub-groups at random, distinctively marked with coloured paint and ear-tagged. They were then bled, weighed and dosed according to markings. The treatments were (a) *red*, copper with other trace elements, (b) *blue*, copper alone, (c) *white*, trace elements alone and (d) *yellow*, controls. The trace element mixture contained iron ammonium citrate, cobalt and manganous sulphates and sodium iodate in such concentrations that a 10 c.c. dose of the solution used supplied 168 mg. Fe, 42 mg. Mn, 28 mg. Co and 14 mg. I. The copper was given as a solution of the sulphate so that a 10 c.c. dose contained 0·5 g. $CuSO_4 \cdot 5H_2O$ or about 125 mg. of the element. For the red group the copper sulphate was dissolved in the trace element solution to the extent of 5 per cent. so that a 10 c.c. dose gave the full amount of copper and other trace elements.

The dosing was carried out regularly each fortnight up to lambing time by one of us (A.H.H.) and the regular gathering for dosing enabled all animals to be kept under veterinary inspection throughout the course of the experiment. Periodic bleedings were made of each ewe in the experiment in October (at tupping time), January, March and May.

The five farms were grouped around the Castleton district and varied in geological formations. Farms A and D were on carboniferous shale stone formation, B and E on limestone, whilst C was partly on shale and partly on limestone. The quality of the grazings varied considerably. Farms A and E were rough moorland and hillside grazings, C and D were somewhat better, whilst the owner of farm B allowed his sheep access to a good field of rape during the winter. No concentrates were fed but during the more severe wintry weather all the farmers fed some home-grown hay, which was generally of indifferent quality. The ewes on farm B were heavier than on any of the other farms, and maintained much better condition.

The breeds of sheep were very varied. The principal ones encountered were Swaledale, Gritstone, Lonk and Kerry Hill, but Border Leicester, Lincoln, White-faced Woodlands and all sorts

of intermediate crosses occurred. Most farmers used Down breed tups, principally Suffolk and Hampshire, in order to get heavier lambs but some of the ewes also showed traces of Down breeds in their ancestry and altogether there was little evidence of any attempt at a definite breeding policy.

The winter was mild for the district, but there were two heavy falls of snow in January and March coincident with the times selected for bleeding, when hay was the sole food of the ewes. The March fall of snow was probably responsible for some of the stillborn and premature births on Farm D, where some ewes were buried in the snow for a day or two.

Lambing commenced towards the end of March, and daily visits were then made to each farm. Each live lamb was ear-tagged shortly after birth and a record number was given to all stillborn lambs. Dosing ceased at lambing time. All cases of swayback were notified. Autopsies were made on those dying from the condition or killed *in extremis* with obvious clinical symptoms: the brain and first few inches of the spinal cord were removed and preserved for subsequent histological examination. It was impossible to conduct a *post-mortem* examination on every lamb that died, but deaths from causes other than obvious swayback were few, and since the farmers were being compensated for every established case of the disease attention was drawn to everything suspicious.

The diagnosis of swayback was based upon a combination of different features. In the acute cases clinical symptoms alone were sufficient and macroscopic evidence of cavitation in the brain was unmistakeable. In the less obvious cases there was histological evidence, and the witness of the serial blood Cu estimations made throughout pregnancy.

The full lambing records are given in Table I, which incorporates the cases of swayback and gives accurate records of other losses encountered amongst ewes in these areas. Only four-fifths of the original number of ewes gave birth to live lambs, and with the wastage through swayback and other causes it will be noted that in the control groups (white and yellow), an owner was fortunate if he was able to raise to maturity one lamb from each ewe.

Considering the summarised totals first, it is apparent that in the copper treated groups six cases of swayback occurred out of 231 lambs born alive (2·5 per cent.), compared with 52 cases from 230 lambs in the groups without copper (22·6 per cent.). Of the latter, 32 were from the yellow group with 112 lambs, and 20 from the white group of 118 lambs, but this difference is not statistically significant, and it may be concluded that trace elements (Fe, Mn, Co, I) other than Cu when regularly dosed to pregnant ewes had no appreciable effect in reducing the incidence of swayback in the offspring. Even with regular fortnightly dosing six cases of the disease occurred showing that at the level administered, Cu does not afford complete protection. These figures will be considered

TABLE I

SUMMARY OF DERBYSHIRE SWAYBACK EXPERIMENTS. MAIN EXPERIMENTAL GROUPS

		Ewes.					*Lambs.*					
									Swayback.			
Farm.	*Group.*	*Total in Group*	*Died.*	*Aborted, Stillborn and Premature Births.*	*Barren, etc.*	*No. Lambed.*	*No. with Swayback Lambs*	*No. Born Alive.*	*No. of Swayback Cases.*	*Singles.*	*One of Twins (one healthy).*	*Twins.*	*One of Twins (one stillborn).*
A	Red ...	31	1	2	3	25	—	38	—	—	—	—	—
	Blue ...	30	—	3	4	23	—	36	—	—	—	—	—
	White ...	31	2	3	5	21	8	34	10	1	4	4	1
	Yellow...	29	—	5	2	22	9	33	13	2	1	8	2
B	Red ...	11	—	—	—	11	—	18	—	—	—	—	—
	Blue ...	10	—	1	—	9	—	16	—	—	—	—	—
	White ...	11	—	1	1	9	—	18	—	—	—	—	—
	Yellow...	10	—	—	—	10	1	17	1	—	1	—	—
C	Red ...	22	2	—	2	18	2	25	2	1	—	—	1
	Blue ...	22	2	—	1	19	2	26	2	1	1	—	—
	White ...	22	1	1	4	16	5	23	7	3	—	4	—
	Yellow...	21	1	3	3	14	4	18	4	3	1	—	—
D	Red ...	20	1	—	3	16	1	22	1	1	—	—	—
	Blue ...	20	1	2	3	14	1	20	1	—	—	—	1
	White ...	20	—	2	1	17	3	28	3	—	3	—	—
	Yellow...	20	1	—	—	19	8	29	11	2	3	6	—
E	Red ...	10	—	—	1	9	—	15	—	—	—	—	—
	Blue ...	10	—	—	—	10	—	15	—	—	—	—	—
	White ...	9	—	1	—	8	—	15	—	—	—	—	—
	Yellow...	12	—	—	2	10	2	15	3	1	—	2	—
Total	Red and Blue	186	7	8	17	154	6	231	6	3	1	—	2
„	White and Yellow	185	5	16	18	146	40	230	52	12	13	24	3
Grand total ...		371	12	24	35	300	46	461	58	15	14	24	5

again in a subsequent publication in which the serial blood Cu findings are discussed, but it may be stated here that all six cases were from ewes with a very low initial Cu status, which failed to show appreciable response to administrations of copper sulphate at the intervals adopted.

With regard to individual farms it will be noted that A, B, and E showed complete protection in the Cu groups. This finding may not be significant with farms B and E where the general incidence was very low, but on farm A the effect is definite. The groups without copper treatment showed the high incidence of 34 per cent. (23 cases from 67 lambs), with no cases at all in the Cu-dosed ewes. On farm C the incidence in the Cu-groups was about 8 per cent. compared with about 27 per cent. in the " controls," and the corresponding figures for farm D were 5 per cent. and 25 per cent. respectively.

Assuming that Cu gave no protection at all, and that the incidence of swayback in treated groups would have been the same as in the control groups, about 104 lambs out of 460 would have been affected. Such an incidence, about 22 per cent. of the total born alive, serves to show the economic importance of the disease in an area where agriculture is already at a low level.

II. DERBYSHIRE : SUBSIDIARY OBSERVATIONS (" LICK " FARMS)

These observations were also conducted in the North Derbyshire area but on farms where, for various reasons, the detailed studies made in the main experimental group could not be carried out. The farmers concerned all used copperised licks of the several commercial

TABLE II

" LICK " FARMS : INCIDENCE OF SWAYBACK

Farms.	*No. of Ewes Lambed.*	*No. of Lambs.*	*Cases of Swayback.*	*Estimated Lick Consumption Oz. per Ewe.*
1.	33	35	—	12
2.	100	100	—	7
3.	49	57	15	28
4.	98	100	1	8
5.	70	110	6	8
6.	45	58	1	28
7.	80	125	2	10
8.	17	28	2	36
9.	39	75	4	31
10.	27	35	—	39
11.	60	84	25	31
12.	58	80	1	14
13.	57	100	1	39
14.	8	10	—	26
15.	30	54	2	27
16.	30	58	14	18
17.	26	36	—	10
18.	13	25	—	105
Total	840	1,170	74	

brands sold in the area. They were asked to supply records of the number of ewes in their flocks, the number of licks (briquettes) offered, and of the number of cases of swayback in the ensuing lamb crop. Periodic visits were paid to each farm throughout the winter and at lambing time. The actual figures presented in Table II are from the farmers' own records.

Table II shows that the incidence of swayback over all the " lick " farms was 74 cases out of 1,170 lambs or about 6·3 per cent. Of these 74, however, 54 occurred on three farms with 199 lambs; an incidence of about 27 per cent. The remainder, 20 out of 971 lambs, or about 2·0 per cent., provide an average incidence for 15 farms similar to the figure of 2·5 per cent. from the Cu-treated animals in the main experimental group. This may be taken as evidence that voluntary consumption of copperised licks is effective on most farms.

The figures for the estimated lick consumption per ewe must, however, be accepted with a great deal of reserve. These are the reported amounts *offered* per ewe, but the influence of weathering and of accessibility must be considered and although it is not possible to allow for these factors considerable wastage must be presumed. The variation is very wide, ranging from 7 to 105 oz. per ewe, or 7 to 39 oz. if the very high figure of farm 18 is left out of account as probably erroneous. Based on the assumption that the licks contained 0·3 per cent. Cu, and reckoning the gestation period as 160 days, the average consumption of about 24 oz. per ewe corresponds approximately to a daily intake of 14 mg. Cu in the licks, an amount sufficient to double the normal daily intake from the herbage. As individual figures show, however, much less than this offered apparent protection on some farms.

It is difficult to account for the high incidence on three farms, Nos. 3, 11 and 16. Farm 3 was adjacent to farm B in the main experimental group (where only one case of swayback was reported) and the alleged consumption of licks was sufficiently high. There is doubtless a wide variation in incidence from farm to farm, and there appears to be some evidence in favour of the view that farmers buying in fresh stock simultaneously disseminate trouble to their neighbours the following year by disposing of their culled ewes. Whilst there is no concrete evidence of a hereditary factor disposing to the disease there is little doubt that a large number of ewes become " conditioned " to a low Cu status by long residence in the area and that these tend to throw an increasing proportion of swayback lambs with advancing age. There is also the possibility that the consumption figures for farms 3, 11, and 16, are unreliable or that the particular batches of licks used were unduly low in copper content.

III. WEYBRIDGE: ATTEMPTED INDUCTION OF SWAYBACK EXPERIMENTALLY

Forty ewes with a known history of swayback from ten different farms were purchased from the Castleton area and tupped on their

own farms. All had delivered swayback lambs in one or more previous years. They were sent to Weybridge early in December and placed on a diet as low in Cu as could be obtained by use of available food-stuffs. The ration was 1 lb. crushed oats, with chaffed oat straw fed up to appetite consumption which averaged about 1 lb. per day. The oats contained 4–8 p.p.m. Cu and the straw 4–6 p.p.m. so that the daily intake of Cu lay between about 4 and 7 mg., probably about 5 mg. on average. Based on a number of analyses the Derbyshire pastures range from about 8 p.p.m. to over 20 p.p.m. so that the absolute Cu intake of grazing ewes could hardly be less than 15 mg. daily and may have been considerably more, *i.e.*, the Weybridge diet was estimated to supply one-third or less of the daily intake of the experimental ewes grazing naturally in the Derbyshire area. This diet is admittedly not balanced nutritionally but the daily protein intake was as good as that estimated for the normal herbage and the fibre probably not as high. Calcium intake was low but in the later stages of experimental feeding a small quantity of bone meal and salt was added and those animals which went back in condition were given a small individual supplement of cod liver oil. Regular live weight determinations were made, and most of the ewes showed increases on the laboratory ration, in contrast to the naturally grazing animals which fell off in condition on the winter herbage and up to lambing time. They were all stall-fed and bedded down on the same type of oat straw as that on which they were being fed in chaffed form.

These 40 ewes were divided into four groups, marked red, white, blue and yellow to correspond with the groups in the field experiment above, and subjected to the same differential treatments of trace elements and copper as in the Derbyshire area. The breeds were as mixed and varied as in the main experiment. The animals themselves were generally very old, by reason of the method of selection, and several were beginning to lose their teeth. They were nearly all in very poor condition on arrival. During the period of late pregnancy several went down in " preparturient hypocalcaemic coma " which, however, responded well to calcium therapy. Some died from diagnosable causes, a few aborted, and five were barren. 28 carried lambs to term and 24 produced live lambs. In all 37 lambs were born but none showed any signs of swayback, and none of the ewes developed lambing sickness. Despite the indifferent quality of the diet and its very low Cu content the surviving ewes were in fair condition and their lambs made extraordinarily good live weight increases. The ration was improved after lambing, when the main purpose of the experiment had been achieved, and the ewes received a good concentrate mixture until enough grass was available to support them. Despite the low Cu content of the diet blood Cu levels rose quickly after arrival at Weybridge and remained normal thereafter. Details are given in Table III.

TABLE III

WEYBRIDGE: EXPERIMENTAL EWES ON LOW COPPER DIET. SUMMARISED OBSERVATIONS

Group.	*Total.*	*Died.*	*Aborted, Stillborn and Premature Births.*	*Barren.*	*Number Lambed.*	*Number of Live Lambs.*
Red ...	10	2	—	2	6	9
Blue ...	10	2	1	—	7	9
White...	10	2	1	2	5	9
Yellow	10	1	2	1	6	10
Total ...	40	7	4	5	24	37

AGE INCIDENCE OF SWAYBACK LAMBS

The 58 lambs with swayback in the Derbyshire experiment showed initial symptoms at varying ages. The majority were affected when born and died within 24 hours of birth, but four were over two months old before symptoms appeared. Table IV shows the actual age incidence. Acute cases are very obvious but with many of the so-called " delayed swayback " cases symptoms are very slight and would pass unnoticed in the ordinary way; but when the flocks are rounded up, and particularly when driven by dogs, slight symptoms are accentuated and the lambs may sway over and have to pause for a few seconds before continuing their incoordinated gait. After resting the symptoms may almost disappear again but in general the disorder becomes progressively worse with each manifestation of incoordination. In many of these chronic cases the lambs may grow normally, and where symptoms are very slight may attain maturity and breed without lapsing into the acute or sub-acute form. It is not unusual to find ewes, in any flock where swayback is endemic, showing slight symptoms of incoordination even after having given birth to several crops of lambs.

TABLE IV

AGE INCIDENCE OF SWAYBACK IN EXPERIMENTAL LAMBS: MAIN EXPERIMENT

Weeks ...	0–1	1–2	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10	Over 10
No. of cases...	31	2	1	6	6	3	4	1	2	1	1

Table IV shows that of the 58 cases of swayback 46 occurred within five weeks of birth, 31 within the first week and 12 after the lambs were five weeks old. The delayed cases are of special interest. Most of them still showed macroscopic brain lesions, although of less extent than in typical acute cases, and all of them came from ewes whose blood Cu values remained low throughout the period of pregnancy. The relation of swayback to blood Cu level will be discussed more fully in a subsequent paper dealing with the analytical protocols.

DISCUSSION

The work of the season 1943–1944 has confirmed the value of copper in preventing swayback in lambs, and has shown conclusively that certain other common trace elements are without effect. By regular dosing it was ensured that each ewe received its full quota of copper instead of being dependent upon methods of supplying copperised licks to the flock. Nevertheless at the dosage given (0·5 g. copper sulphate each fortnight) prophylaxis was not perfect, and it would appear that for some individual animals higher or more frequent dosage is necessary in order to afford complete protection. Where labour conditions permit it is more satisfactory to dose susceptible ewes at regular intervals than to rely upon voluntary consumption of exposed licks, but since copper is poorly absorbed from the gut it is considered that smaller doses at short intervals are better than large doses less frequently. 2 g. copper sulphate is about the safe upper limit for a single dose to a ewe of average weight so that the actual dose given each fortnight was well below the possible danger point. Routine monthly anthelmintic treatment with 100 c.c. of a 1 per cent. solution of $CuSO_4 \cdot 5H_2O$ was widely practised in some areas before the introduction of phenothiazine, and it is believed that this incidentally reduced the prevalence of swayback on affected farms. But for specific control of the disorder we think the interval should not exceed a fortnight. Obviously the best treatment would be a small dose every day. That is what " licks " aim at supplying, and their only disadvantage is the uncertainty of consumption. It would be impracticable for most farmers to dose more often than once a fortnight throughout the pregnancy period, which incidentally coincides with the time when weather conditions are most severe. Monthly dosing, even if it could be shown to be sufficiently protective, suffers from the disadvantage that a complete gather of the sheep is not always possible and individual sheep might miss one or more of the five doses intended, even the two most critical doses in the later stages of pregnancy.

For farmers unable or unwilling to adopt routine dosing, the amount of copper sulphate in commercial licks could be safely raised from the 0·3 per cent. now in vogue, to 1 per cent. or even 2 per cent. This would increase the measure of protection on some types of farm.

SUMMARY

Experimental work on swayback has been continued by field experiments in the North Derbyshire area, testing specifically the effects of copper and other trace elements administered orally to pregnant ewes, by observations on the efficacy of copperised licks, and by attempted induction of swayback in susceptible ewes fed on a ration low in copper.

The main dosing experiment was carried out on five farms of varying geological formation, with 371 ewes divided into groups, receiving copper and a trace element mixture (cobalt, iodine, iron and maganese) separately and combined, at fortnightly intervals throughout pregnancy. Full lambing records are given. Of 231 lambs from ewes given copper alone or with other trace elements only six developed swayback, whereas 52 cases of the disease occurred in 230 lambs given trace elements alone or left as controls. Copper sulphate at the level of 0·5 g. once a fortnight afforded a high measure of protection but other trace elements were without significant effect.

A survey of 18 farms representing 840 ewes allowed access to commercial anti-swayback licks containing copper showed an incidence of 74 cases of swayback from 1,170 lambs, the majority being found on three farms. Whilst copper offered in lick form gave a high degree of protection on most farms serious breakdowns occurred, possibly because of failure to consume the licks.

An experimental group of 40 ewes from the Derbyshire area, selected as having given birth to swayback lambs in previous years, were transferred to Weybridge and fed on a ration supplying about 5 mg. Cu daily, less than one-third of that estimated to be consumed by the sheep in their natural habitat. They were divided into groups and differentially dosed as in the main Derbyshire experiment, but no cases of swayback were encountered in the resultant lamb crop. This indicates that low daily intake of copper is not the only factor in the etiology of the disease.

The age incidence of swayback in Derbyshire is given. Although the greater proportion of cases occurred during the first few days of life a number of lambs, apparently normal at birth, developed the disease when two to three months old. Systematic blood analyses conducted throughout the investigations are discussed separately in part II of this report.

ACKNOWLEDGMENT

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REFERENCES

Bennetts and Chapman. (1937). *Aust. vet. J.*, **13,** 138.
Dunlop and Wells. (1938). *Vet. Rec.*, **50,** 1175.
Dunlop, Innes, Shearer and Wells. (1939). *J. comp. Path.*, **52,** 259.
Innes and Shearer. (1940). *ibid.*, **53,** 1.
Shearer, Innes and McDougall. (1940). *Vet. J.*, **96,** 309.

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CONTRIBUTIONS TO THE STUDY OF SWAYBACK IN LAMBS

II. BLOOD COPPER INVESTIGATIONS

By

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INTRODUCTION

The work of Bennetts and Chapman (1937) demonstrated the role of Cu in the aetiology of " swayback " in lambs in Western Australia and these workers were the first to show that susceptible ewes and affected lambs had a very low blood copper status. Further elaboration of these findings from the biochemical aspect was made by Beck (see Bennetts and Beck, 1942), who correlated the low Cu status of the animal with that of the herbage in areas where swayback is endemic.

In this country extensive biochemical studies were carried out by Innes and Shearer (1940) and by Shearer, Innes and McDougall (1940) in Derbyshire where swayback is the most serious of the diseases of lambs. These investigators demonstrated that low levels of blood Cu were to be found in the pregnant ewes, but, in contrast to Australia, the pastures contained apparently normal amounts of Cu. During the season 1943–44 further observations on the general swayback problem of Derbyshire were made by the present collaborators (Hunter, Eden and Green, 1945), investigating more extensively the role of Cu in the disease, and the present communication sets forth the results of blood studies ancillary to the field investigation.

Shearer *et al.* showed that the level of blood Cu in ewes in Derbyshire lay between 0·02 and 0·03 mg.p.c. in November, rising throughout January to April when figures of about 0·07 mg.p.c. were reached. This, they considered, was a rise associated with pregnancy as reported by Tomsett and Anderson (1925) for the human subject, although they failed to find any indication of a pregnancy rise in sheep with initially normal levels of blood Cu. Absence of variation during pregnancy was also borne out by investigations on normal sheep at Weybridge (Eden, 1941a).

I. DERBYSHIRE INVESTIGATIONS : MAIN EXPERIMENTAL AREA

In the present series of experiments we have had the advantage of larger numbers of sheep under controlled conditions and it has been possible to make serial blood studies on a scale not afforded to the previous workers. 371 ewes on five farms were divided into four groups (a) Cu-treated, receiving 0·5 g. copper sulphate in solution each fortnight, with and without other trace elements and (b) non-Cu groups, receiving either trace elements other than Cu or acting as

controls. The incidence of swayback in group (a) was 6 cases from 231 lambs and in (b) 52 cases from 230 lambs, a difference significantly in favour of Cu treatment. These results have already been discussed in Part I of this report.

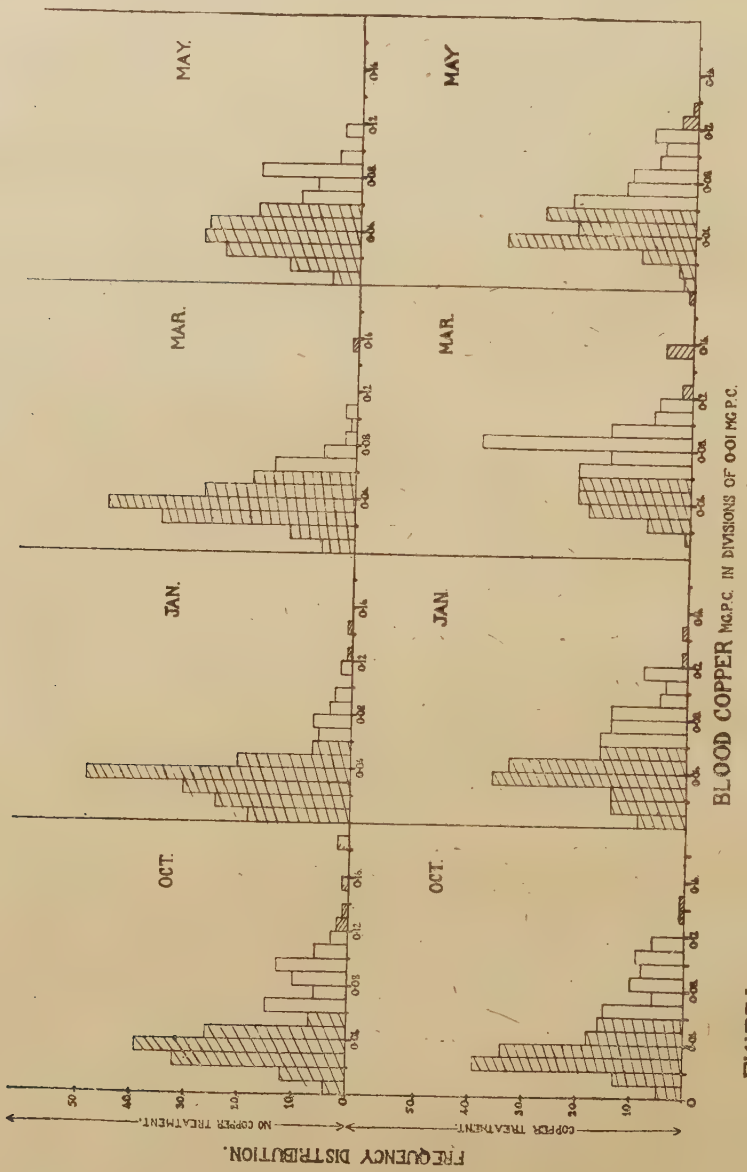

FIGURE I.

Each ewe was ear-tagged in October and blood samples were analysed for Cu by the method of Eden and Green (1940). A second bleeding was made in January, eleven weeks later, and a third after

another seven weeks just before lambing began in March. A final bleeding was made at the end of May eleven weeks after the March samples had been taken, and on Farm A a further representative selection of ewes was bled in July to give summer blood Cu values.

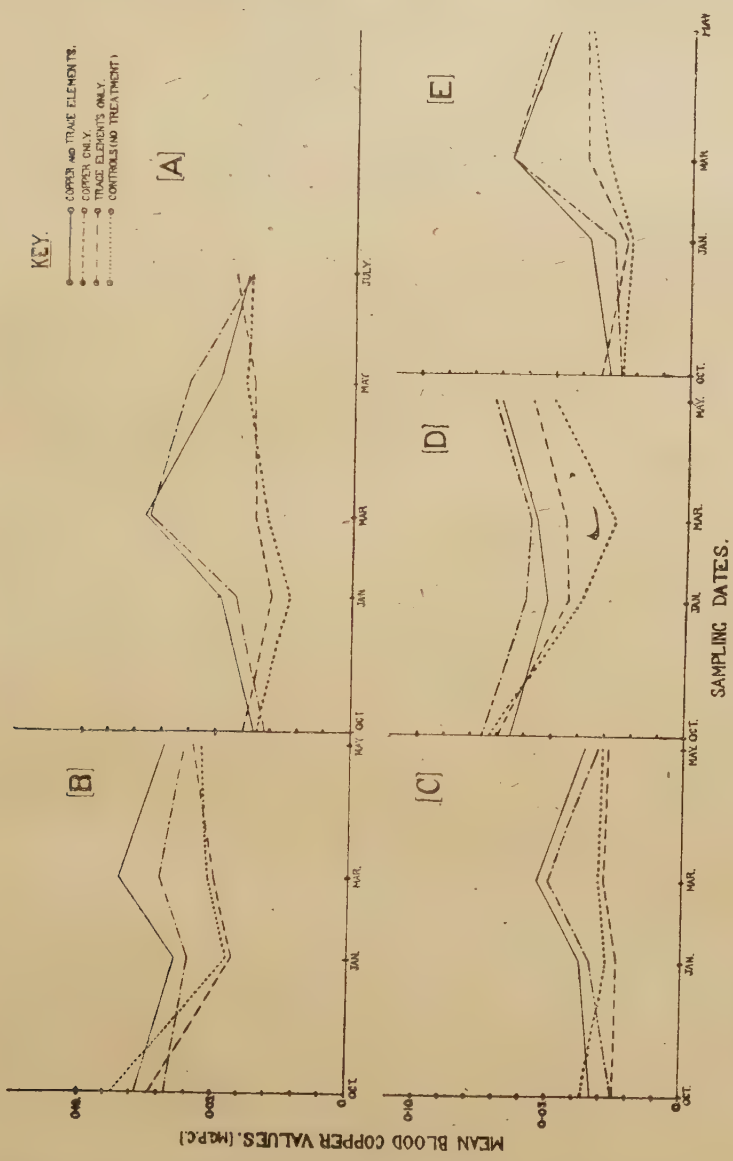

FIGURE II.

Although one or two animals were missed at each gather and a few deaths occurred over the winter, it was thus possible to procure serial values for each ewe in the experiment.

The data are too numerous to detail individually but are considered, firstly, *en masse* in a series of histograms in Figure I and, secondly, as group averages on each farm presented graphically in Figure II, with more detailed exposition of the values from those ewes which gave rise to swayback lambs.

In Figure I the data are arranged at 0·01 mg.p.c. intervals and divided into two main groups, copper treated and controls. Areas are shaded below 0·06 mg.p.c. and above 0·12 mg.p.c. The October copper values were taken before treatment differentiations were made, and of the 366 samples suitable for analysis 245 (or 67 per cent.) lay below 0·06 mg.p.c. and only eight exceeded 0·12 mg.p.c. Of the values below 0·06 mg.p.c. 34 were less than 0·02 mg.p.c. and nine were less than 0·01 mg.p.c. 144 values lay between 0·02 and 0·04 mg.p.c. so that nearly 49 per cent. of the total were below 0·04 mg.p.c. The general average of all the values was about 0·046 mg.p.c. The distribution of the two groups was essentially similar.

By January, in contrast to Shearer's findings, the general average of all observations had fallen slightly to 0·039 mg.p.c., despite Cu treatment of half the ewes. Of the 185 Cu-treated animals, 122 (66 per cent.) had values below 0·06 mg.p.c., with 23 below 0·02 mg.p.c.; only two exceeded 0·12 mg.p.c. Of the 176 controls 152 (86 per cent.) lay below 0·06 mg.p.c., 19 values being less than 0·01 mg.p.c., whilst again only two exceeded 0·12 mg.p.c.

Before lambing in March the general improvement in blood Cu level of the Cu-treated groups was definitely apparent. Only 70 of the 180 Cu-treated ewes had values below 0·06 mg.p.c. (39 per cent.) and 7 exceeded 0·12 mg.p.c., the greater proportion lying between 0·06 and 0·12 mg.p.c. and approaching what may be regarded as almost normal levels; only one ewe had a value less than 0·02 mg.p.c. In the control groups about 85 per cent. lay below 0·06 mg.p.c. (147 out of 174) a figure of the same order as in the January bleeding. 100 of these lay below 0·04 mg.p.c. and 18 were below 0·02 mg.p.c. The general mean of all the ewes in March was 0·051 mg.p.c.

Lambing commenced towards the end of March and the fortnightly dosings then ceased. The final bleeding was carried out towards the end of May when lambing had been completed and most of the lambs were well over a month old. The general mean had then dropped slightly to 0·049 mg.p.c. and the values of the Cu-treated ewes tended to fall to the same levels as the controls. Of the 173 Cu-treated ewes 100 lay below 0·06 mg.p.c. (58 per cent.) compared with 119 of 163 controls (73 per cent.); only four in the former group and none in the latter exceeded 0·12 mg.p.c.

Considering all the May figures together about 65 per cent. lay below 0·06 mg.p.c. and 1·2 per cent. exceeded 0·12 mg.p.c. The corresponding initial figures at the October bleeding were 67 per cent. and 2·2 per cent. and the means 0·046 and 0·049 mg.p.c. respectively.

Reviewing the whole series of bleedings there is no evidence from the mass data presented that there is any pronounced rise of blood Cu during pregnancy *per se* and the rise in the Cu-treated ewes must be accepted as being entirely due to the effects of regular dosage with Cu. This confirms previous observations on the blood Cu status of ewes in relation to pregnancy made on ewes in Northumberland and at Weybridge (Eden, 1941 a and b).

Blood Cu variations associated with individual farms.

The individual numbers of ewes on each farm are too small to allow of the same form of histogram treatment as the combined mass of data, but some conception of the trends on each farm can be obtained by a consideration of the averages of each group at the different bleedings, comparing the Cu-treated and control groups on each farm and the combined farms treated as a whole.

These figures are given in Table I, and are shown graphically in Figure II.

TABLE I

AVERAGE BLOOD CU VALUES ON EACH FARM AT EACH BLEEDING. BLOOD COPPER MG. PER CENT.

Farm.	*Copper Treated.*				*No Copper Treatment.*			
	Oct.	*Jan.*	*Mar.*	*May.*	*Oct.*	*Jan.*	*Mar.*	*May.*
A	0·034	0·046	0·076	0·056	0·037	0·027	0·034	0·039
B	0·072	0·062	0·077	0·065	0·080	0·044	0·051	0·056
C	0·029	0·036	0·052	0·034	0·031	0·026	0·030	0·030
D	0·071	0·056	0·057	0·070	0·069	0·042	0·036	0·054
E	0·028	0·034	0·068	0·050	0·029	0·024	0·035	0·040
Mean	0·045	0·046	0·066	0·054	0·047	0·034	0·036	0·043

On Farm A the blood Cu status was initially low, ranging in the four groups from 0·032 to 0·040 mg.p.c. By January the Cu-treated ewes were beginning to show elevated Cu values whilst the values for the control ewes had fallen, and by March the two Cu-treated groups had values of 0·076 and 0·077 mg.p.c. as compared with control values of 0·032 and 0·036 mg.p.c. It is interesting to note that on this farm Cu therapy gave complete protection from swayback and that the incidence of the disease in the control groups was about 34 per cent., the highest in this season's observations. By May, six weeks after cessation of treatment, the values for the Cu-treated groups had fallen appreciably whilst the controls had regained their initial October values.

On Farm E the difference between the Cu-treated and control groups was also pronounced at the March bleeding, and by May the values were again approaching each other. Here again Cu therapy gave complete protection, although the incidence of swayback in the two control groups was very low despite their low Cu status in March (0·031 and 0·039 mg.p.c.).

The Cu-levels of the ewes on farm B were initially fairly high, ranging from 0·067 to 0·087. There was a general fall in January but by March improvement was shown, with a definite difference in favour of the Cu-treated groups. Incidence of swayback on this farm had been very high the previous year but during the present investigation only one case was observed (in a control group). The standard of farming here was much higher than on any other farm, and the ewes were in first-class condition throughout the winter mainly because of the fact that they had access to a good field of rape and received liberal allowances of fair quality hay during the worst part of the winter.

On farm C four cases of swayback occurred in the Cu-treated groups compared with 11 in the controls. In October the blood Cu status of all the ewes was very low and the average for the control groups remained low throughout the investigation. The levels of the Cu-treated groups showed some improvement by March, with an average of 0·052 mg.p.c. as compared with 0·030 mg.p.c. for the controls. This March average of 0·052 mg.p.c. was the lowest of any of the Cu-treated groups on all farms and was about the same level as the controls on farm B. Quite a number of ewes receiving Cu on this farm failed to show any pronounced alteration of their blood Cu levels; special consideration will be given to these later.

Two cases of swayback occurred in Cu-treated groups on farm D compared with 14 in the control groups. In October the averages for blood Cu of the four groups ranged from 0·065 to 0·076 mg.p.c. By January these figures had dropped considerably and in March the two Cu-treated groups had averages of 0·056 and 0·058 mg.p.c., compared with 0·027 and 0·045 mg.p.c. in the controls. Following lambing there was an all-round improvement, the Cu-treated groups returning to their October figures and the controls rising to 0·050 and 0·058 mg.p.c.

Blood Cu Records of Ewes Producing Swayback Lambs

So far consideration has been given to mass distribution trends in blood Cu for the two main treatments, and also to the average values for these on the individual farms. It is now interesting to examine the blood records of the individual ewes which gave birth to swayback lambs; these are given in Table II for the 46 ewes concerned.

Comparing first the averages of the ewes in Table II which produced swayback lambs, with the general mass of both Cu-treated and control ewes in Table I, it will be noted that their values are much lower. The March values are the most interesting as they reflect the position in the later stages of pregnancy; the mean value of 0.025 mg.p.c. (including the six Cu-treated "swayback" ewes) is well below the average even of the controls (0.036 mg.p.c.).

TABLE II

SERIAL BLOOD COPPER STUDIES ON EWES BEARING SWAYBACK LAMBS.

BLOOD Cu IN MG. PER CENT.

Group.	*Farm.*	*No.*	*Oct.*	*Jan.*	*Mar.*	*May*	*Group.*	*Farm.*	*No.*	*Oct.*	*Jan.*	*Mar.*	*May.*
Red ...	C	217	0·01	<0·01	0·02	0·02	Yellow...	A	24	0·03	0·02	0·05	0·04
..	..	242	<0·01	<0·01	0·02	<0·01		..	44	0·06	0·03	0·02	0·04
	D	269	0·02	0·01	0·03	0·04		..	48	0·03	0·02	0·03	0·03
Blue ...	C	171	0·07	0·01	0·02	0·03		..	52	0·03	0·01	0·02	0·06
..	..	228	0·01	0·02	0·03	0·02		..	64	0·03	0·03	0·03	0·02
	D	287	0·02	0·01	0·03	0·01		..	72	0·03	0·03	0·03	0·04
White ...	A	2	0·04	0·02	0·02	—		..	84	0·04	0·03	0·03	0·03
..	..	26	0·03	0·09	0·05	0·04		..	92	0·03	0·03	0·01	0·02
..	..	30	0·12	0·03	0·03	0·07		..	100	0·02	0·01	0·03	0·04
..	..	46	0·03	0·02	0·02	0·02		B	138	0·09	0·03	0·06	0·05
..	..	74	0·02	0·02	0·02	—		C	229	<0·01	0·01	0·02	<0·01
..	..	78	0·03	0·03	0·02	—		..	233	—	<0·01	<0·01	0·01
..	..	86	0·02	0·01	0·01	0·01		..	241	0·01	<0·01	0·02	0·03
..	..	98	0·05	0·03	0·03	0·06		..	370	—	0·03	0·03	0·02
	C	170	0·02	0·02	—	0·02		D	260	0·09	0·07	0·02	0·02
..	..	178	0·02	0·03	0·04	0·08		..	276	0·05	0·04	0·03	0·03
..	..	222	0·02	0·02	0·02	0·03		..	280	0·04	0·03	0·02	0·06
..	..	246	0·02	0·03	0·02	0·01		..	284	0·04	0·01	0·03	0·05
..	..	372	0·02	0·04	0·03	0·01		..	288	0·03	<0·01	0·03	0·04
	D	274	0·06	0·01	0·03	0·03		..	292	0·04	0·01	0·03	—
..	..	294	0·06	0·03	0·04	0·02		..	312	0·05	0·02	<0·01	0·01
..	..	318	0·03	<0·01	—	0·02		..	320	0·02	0·03	<0·01	0·02
								E	335	0·02	0·03	0·02	0·02
								..	339	0·03	<0·01	0·02	0·05

	Oct.	*Jan.*	*Mar.*	*May.*
Means:	0·035	0·023	0·025	0·030

Key: *Red* = Cu + Trace Elements. *Blue* = Cu. *White* = Trace Elements *Yellow* = No treatment.

The six Cu-treated ewes with one exception (No. 171) all had low initial values, which either failed to improve or only increased very slightly over the pregnancy period despite Cu treatment. Evidence from Table I of the value of Cu prophylaxis in improving the blood Cu status of animals with initially low levels is clear even although Table II shows that in certain individual cases the level of Cu dosing adopted was completely without effect. The records of the season show that there were a few more ewes whose blood picture failed to respond to Cu treatment, but which nevertheless gave birth to apparently normal lambs.

Considering the March values of all the ewes which produced affected offspring, the highest level was 0·06 mg.p.c. in ewe 138 of farm B (where the best average figures were obtained all through). Of the 44 available March values only 5 are of 0·04 mg.p.c. or above, and 22 (50 per cent.) lie at 0·02 mg.p.c. or less. There is thus a close correlation of low blood Cu values just prior to lambing, with incidence of swayback. On the other hand 132 out of 241 ewes which produced normal live lambs had March levels of 0·05 mg.p.c. or less, and 27 of these had values of 0·02 mg.p.c. or below. It can be reasonably concluded from these observations that whereas swayback in the lamb is definitely connected with a low preparturient blood Cu level of the mother, a low level seen in the ewe does not necessarily imply the condition of swayback in the resulting offspring. Hence blood analyses of individual pregnant ewes are of little value for diagnosis of prospective swayback in the offspring, although serial blood Cu studies are useful in forecasting the probable incidence in a flock and as confirmatory aids to diagnosis. Results will be presented later to show that the same type of correlation holds true for lambs on swayback farms.

Little can be said at this stage of predisposing causes of swayback except that they are correlated with the Cu status of the flock, and that there is obvious need for more fundamental studies of the physiology of Cu in relation to the development of the foetus.

II. WEYBRIDGE INVESTIGATIONS

As stated in Part II, the transference of 40 ewes of known swayback history from Derbyshire to Weybridge and their maintenance in stalls on a basal diet of oats and oat straw very low in copper, did not result in a single case of swayback in the resultant lamb crop. The ewes were divided into four groups of ten, (Cu treated, with and without other trace elements, controls, with and without trace elements), along the same lines as in the main Derbyshire experimental programme. Some of the ewes were bled for Cu observations before leaving the home farms on which they had been tupped, and all were bled on arrival at the laboratory on December 7th and at frequent intervals subsequently. For various reasons some ewes died before lambing, others were barren, but of the original number, 31 were available for bleeding throughout the winter and after lambing. The results are given in

Table III where the term "post-parturient" refers to samples taken immediately after lambing, or at the beginning of May when it was certain that some of the ewes were barren.

TABLE III

BLOOD COPPER RECORDS OF DERBYSHIRE EWES TRANSFERRED TO WEYBRIDGE. BLOOD COPPER IN MG. PER CENT.

Group.	*No.*	*Nov. 27th.*	*Dec. 7th.*	*Dec. 28th.*	*Jan. 18th.*	*Feb. 14th.*	*Post-Parturient.*	*Lambing Record.*
Red (Cu)	9957	—	0·03	0·08	0·11	0·09	0·16	Barren
	9965	—	0·04	0·09	0·12	0·07	0·11	Single
	9969	—	0·02	0·08	0·12	0·11	0·13	Twins
	9985	0·04	0·06	0·08	0·12	0·08	0·13	„
	9989	0·01	0·02	0·16	0·11	0·11	0·15	„
	9993	0·02	0·02	0·08	0·13	0·09	0·12	Single
	9999	0·05	0·02	0·09	0·11	0·08	0·11	„
	Mean	(0·030)	0·030	0·094	0·117	0·090	0·130	
White (No Cu)	9954	—	0·05	0·08	0·09	0·09	0·12	Twins
	9958	—	0·02	0·04	0·10	0·06	0·10	Barren
	9962	—	0·03	0·08	0·13	0·09	0·12	„
	9966	—	0·05	0·08	0·13	0·08	0·15	Twins
	9978	0·08	0·09	0·07	0·12	0·11	0·11	„
	9982	0·04	0·05	0·08	0·11	0·09	0·10	Single
	9986	0·01	0·03	0·07	0·11	0·08	0·11	Twins
	9994	0·01	<0·01	0·08	0·09	0·09	0·13	Single (born dead)
	Mean	(0·035)	0·041	0·072	0·110	0·086	0·118	
Blue (Cu)	9959	—	0·04	0·09	0·12	0·11	0·08	Single
	9963	—	0·04	0·09	0·09	0·11	0·13	„
	9967	—	0·03	0·09	0·11	0·08	0·14	Twins
	9979	0·05	0·08	0·08	0·13	0·11	0·15	Single
	9987	0·03	0·02	0·09	0·12	0·08	0·11	„
	9991	0·02	0·02	0·10	0·13	0·08	0·15	Twins
	9995	0·04	0·03	0·14	0·12	0·11	0·13	Single
	Mean	(0·035)	0·037	0·097	0·117	0·097	0·127	
Yellow (No Cu)	9956	—	0·02	0·06	0·10	0·07	0·12	Twins
	9960	—	0·03	0·08	0·10	0·07	0·13	Single
	9964	—	0·03	0·07	0·15	0·07	0·12	Twins
	9968	—	0·06	0·09	0·11	0·10	0·11	Barren
	9972	—	0·04	0·07	0·10	0·08	0·11	Twins
	9984	0·02	0·05	0·08	0·09	0·07	0·12	Single (born dead)
	9988	0·02	0·02	0·05	0·12	0·08	0·12	Twins (dead one)
	9992	0·02	0·02	0·05	0·12	0·04	0·11	Single
	9996	0·02	<0·01	0·07	0·13	0·08	0·12	Twins (born dead)
	Mean	(0·020)	0·030	0·069	0·113	0·073	0·118	

The most interesting features of the blood records in Table III are the low Cu levels of the ewes, both in Derbyshire and on arrival at Weybridge, and their rapid rise within three weeks (7th

to 28th December) to what may be regarded as more common normal levels, in spite of the very low Cu content of the diet and irrespective of dosage with Cu. Once again there is no evidence of any material rise associated with pregnancy *per se*, apart from that of environmental change. The Cu-dosed groups have slightly higher average levels than those untreated, statistically insignificant but perhaps bearing out previous findings (Eden, 1939) that regular dosage with copper sulphate has little material effect on blood Cu levels when these are " normal." The ewes were stall-fed throughout pregnancy and had no access to alien sources of Cu. Despite the selected experimental ration, assessed to supply no more than one-quarter to one-third of the daily intake of copper of the naturally grazing Derbyshire sheep, it is evident from the blood data, either that the small amount of copper it contained was highly available to the ewes or that the natural herbage contained some factor which, in a manner unknown, depresses the normal blood copper level. In Derbyshire the blood copper values remained low or fell lower on grass containing 8 to 20 p.p.m. Cu (dry matter basis). On the Weybridge ration, which contained only 5 p.p.m., they rose rapidly and remained high.

DISCUSSION

The low Cu status of many of the sheep in the Derbyshire area, first reported by Shearer and co-workers has been confirmed although we have been unable to agree with their findings that the blood Cu level rises with advancing pregnancy. The present work has shown that unless Cu supplements are given the values tend to remain low throughout pregnancy, but that regular fortnightly dosing with 0·5 g. of copper sulphate effects a general rise to more " normal " levels although certain individual ewes fail to show improvement. Low Cu values in ewes are closely correlated with the development of swayback in the offspring, although this correlation is not perfect. Swayback has been found to occur in lambs from ewes whose pre-parturient blood Cu level was as high as 0·06 mg.p.c., whilst normal lambs have been born from ewes with values below half this figure.

From the now extensive literature on blood Cu values from various parts of the world it is evident that wide variations can occur in apparently normal ewes, and it is difficult to define exactly what is implied by a " normal " blood level for Cu, but from all the evidence it appears that values below 0·06 mg.p.c. can fall outside the common physiological range. Such values may then be regarded as " low " in the general sense of the term. Values below this figure have not been found in hundreds of analyses from sheep at Weybridge, or in lambs from North Wales except on one particular farm where observations have been recently recorded (Eden, 1944).

From our own observations and from those reported by Shearer *et al.* the Derbyshire pastures are not Cu deficient in the absolute sense of the term. Yet there is the interesting observation, first

made in lambs from North Wales and substantiated by this year's work on Derbyshire ewes, that when animals with low blood Cu are transferred to Weybridge, on to diets of even lower Cu content, blood Cu levels immediately begin to rise to the normal values for the area. In connection with the swayback area of Derbyshire it has for some years been the custom for many flock masters to winter their ewes away in parts of Cheshire, Staffordshire and Notts, as a means of preventing swayback in the resulting lamb crop, and it is probable that such transfers result in rise of blood Cu levels sufficient to tide the ewes over the pregnancy period.

So far there is no explanation for the anomalous observation of low blood Cu levels apparently associated with adequate Cu content of the pastures. Cu appears to be specific in its prophylactic effect, and the disease in its more acute manifestations appears to be associated with the intrauterine development of the lamb although delayed cases, extending up to three months before clinical symptoms are shown, are not unknown. The condition appears to be associated with specific areas of the country and the possibility cannot lightly be dismissed that some factor is present which either renders the copper in the pasture unavailable in digestion, or specifically interferes with the intermediary metabolism of copper in the ewe.

SUMMARY

Serial blood Cu studies were carried out on about 370 ewes in Derbyshire, distributed over five farms, throughout the period of pregnancy, in connexion with a large scale field experiment on swayback in lambs. For the purpose of considering the effect of prophylactic treatment on blood Cu levels, the four groups in Part I of this report are reduced to two; those dosed orally with 0·5 g. of copper sulphate once a fortnight and those receiving no copper.

The Cu status of the ewes in October was very low, the mean blood value being 0·051 mg.p.c. Two-thirds of the animals showed figures below 0·06 mg.p.c., with practically half below 0·04 mg.p.c. In the groups receiving no Cu the blood values had by January fallen even lower, 86 per cent. lying below 0·06 mg.p.c., and just before lambing in March the distribution was much the same. The Cu-treated groups showed slight improvement in January although 66 per cent. of the figures were still below 0·06 mg.p.c., but by March their values had markedly improved and only 39 per cent. lay below 0·06 mg.p.c. Cu treatment ceased after lambing in March, and by May the elevated values had fallen almost to the level of the controls. A supplementary blood sampling carried out on the largest farm in July showed that the previous differences between the groups had vanished.

The improved Cu status of the treated ewes was reflected in the much lower incidence of swayback in the resulting lamb crop,

as compared with ewes receiving no copper. In general those ewes which gave rise to swayback lambs had a much lower preparturient blood Cu level than those which produced normal lambs, although a low level in an individual mother did not necessarily imply the condition of swayback in her offspring.

Derbyshire ewes with a low blood Cu status transferred to Weybridge and maintained on a basal ration of oats and oat straw very low in copper showed a rapid improvement in blood Cu levels and this was reflected in the absence of swayback amongst the offspring. It would seem that some factor in the natural pastures in Derbyshire, other than mere quantity of Cu, is responsible for the low blood Cu status in ewes grazing thereon, and is closely associated with the development of swayback in lambs.

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REFERENCES

Bennetts and Chapman. (1937). *Austr. vet. J.*, **13,** 138.
Bennetts and Beck. (1942). *Counc. sci. ind. Res. Austr. Bulletin* No. 147.
Eden. (1939). *J. comp. Path.*, **52,** 249.
Eden and Green. (1940). *Biochem. J.*, **34,** 1202.
Eden. (1941a). *Ibid.*, **35,** 813; (1941b). *J. agric. Sci.*, **31,** 186; (1944). *J. comp. Path.*, **54,** 220.
Hunter, Eden and Green. (1945). *Ibid.*, **55,** 19.
Innes and Shearer. (1940). *Ibid.*, **53,** 1.
Shearer, Innes and McDougall. (1940). *Vet. J.*, **96,** 309.
Tompsett and Anderson. (1935). *Brit. J. exp. Path.*, **16,** 67.

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OVINE PARATUBERCULOSIS (JOHNE'S DISEASE OF SHEEP)

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INTRODUCTION

Johne's disease has long been known to be transmissible to sheep, and naturally occurring cases of the disease in these animals have been reported from Europe, India and North America.

M'Fadyean (1916), and later, Dunkin (1934), suggested that the disease might be more widespread in this country than was realised and Wilson (1934) has recorded the comparative frequency with which Johne's disease in sheep may be encountered in Scotland.

The earlier workers, Twort and Ingram (1913) and M'Fadyean (1916), in this country, and Howarth (1932) in North America, experienced no difficulty in cultivating the causal organism on suitable media, but more recently Dunkin and Balfour-Jones (1935) found that it grew in primary culture only after a long period of incubation while McEwen (1939), using a variety of different media, was unable to obtain growth even after incubation for periods up to 12 months.

It is the purpose of this paper to confirm the implication in the work of previous authors that there are two closely related organisms responsible for Johne's disease of sheep.

PRESENT INVESTIGATION

Within recent years Johne's disease has been diagnosed at Moredun Institute in sheep from most of the border counties of Scotland and also from the Lothians, Lanark and Sutherland. Many of these animals came from hill flocks, and in no instance was there a history of the sheep having been associated with cattle affected with chronic enteritis. Commonly the disease would appear to be sporadic in its occurrence, only one or two cases appearing among a flock, but on other farms several "pining" animals may be lost every year. Only rarely have cases been seen in animals under the age of three years, the majority occurring in ewes of three years and over and in these animals clinical symptoms are usually first observed in late winter and spring.

The clinical signs are those of a severe debilitating disease. The animal loses condition, the wool is loosened, emaciation develops and death generally supervenes within two to three months. Diarrhoea is a remarkably inconstant symptom and may not be observed although often present intermittently. Diagnosis during life may not be easy; numerous acid-alcohol fast organisms conforming to typical *M. johnei* may be present in the faeces, but the number may not always be sufficiently large to warrant a definite diagnosis, and the results obtained with avian tuberculin and with johnin are of

doubtful value. As is shown elsewhere (Stewart, McCallum and Taylor, 1944), the biochemical examination of blood and the demonstration of a fall in blood magnesium may be of considerable assistance in establishing a diagnosis.

At autopsy, the carcase of an animal which has shown clinical symptoms of the disease is generally emaciated and there may be an almost complete absence of body fat; otherwise the lesions are confined to the intestine and mesenteric lymph nodes which are oedematous and may be very much enlarged. Extensive lesions in the bowel have been found, however, on two occasions even in animals in very good bodily condition, slaughtered for other purposes. Lesions in the intestine may be confined to the posterior part of the ileum, but nearly the whole of the small and large bowel may be affected.

Macroscopically, the lesion consists of a thickening of the mucous coat, but in some cases this may be so slight as to be almost unnoticeable. Corrugations such as are seen in the bovine disease may or may not be present, but are never so prominent as in cattle. As was described by Wilson (1934) the mucous surface of the affected bowel in the majority of cases is stained yellow with bile and in some animals may even assume a deep orange colour.

Microscopically, smear preparations from the affected bowel usually show enormous numbers of acid-alcohol fast organisms and may even present the appearance of a culture film containing a few cells. The pathological histology of Johne's disease in cattle is well known and in sheep has been described at some length by McEwen (1939), who confirmed the opinion of earlier workers that the lesions were essentially similar to those found in cattle.

CULTIVATION OF THE ORGANISM

At Moredun Institute, prior to 1939, numerous attempts to cultivate the acid-alcohol fast organisms associated with the lesions of Johne's disease in sheep had proved entirely negative. Since that date different media have been inoculated with material from 15 affected sheep from eleven farms. From four of these animals, all from the same farm, an organism apparently identical with *M. johnei* (*M. paratuberculosis*, Johne's bacillus) was isolated without difficulty, but from the remainder growth was either not obtained or was very slight.

Materials and Methods.—The most suitable medium has been found to be that described by Minett (1942) containing whole egg and liver extract with 4 per cent. of glycerine, 1 per cent. of heat killed *M. phlei*, and a suitable concentration of gentian violet. Latterly, the dye has been omitted, and it has been found advisable to autoclave the phlei suspension before incorporating it in the medium in order to ensure the death of the organisms. Numerous modifications of this medium have been tried. Egg yolk has been

used instead of whole egg, with or without the addition of liver extract; serum and/or potato extract have been added, the proportion of glycerine has been varied and tuberculin or finely minced affected sheep bowel has been employed in place of *M. phlei*. The media were distributed and inspissated in screw-cap bottles or test tubes which after inoculation were sealed with paraffin wax or rubber caps.

Infected material has been treated with antiformin or 5 per cent. oxalic acid prior to inoculation or in some cases inoculated direct after aseptic dissection through the peritoneal surface of the bowel. Lymph node has also been inoculated direct. The cultures have been incubated at 37° C. to 38° C. for periods up to two years.

In order to control the suitability of the media for *M. johnei*, several bovine strains were isolated and maintained throughout the investigation.

Results.—Of the 15 cases from which media were inoculated a normal growth of *M. johnei* was obtained from four. These four cases all came from the same farm and presented features at autopsy which varied somewhat from the remainder. The affected bowel in each was greatly thickened but without marked pigmentation, and in three instances organisms were only found with difficulty. Films from the intestine of the fourth sheep showed bacilli which, although numerous, were not in such numbers as are usually associated with the ovine disease. Media inoculated from these animals showed growth which was visible to the naked eye after four weeks' incubation and at the end of eight weeks was almost profuse. The primary cultures were subcultivated on to plain egg, glycerine egg and glycerine agar as well as on to a *phlei* medium. Subcultures grew only on the last, and it was therefore assumed that the organism was of the classical *M. johnei* type.

In the remaining cases, growth either did not occur or was observed as an increase in the number of organisms present in films, and even this evidence of multiplication was not demonstrable until after incubation for from 12 to 16 months. Subcultivation at this stage did not lead to an acceleration of growth but merely to a further increase in the number of bacilli present in films after a similar period of incubation. Prolongation of incubation up to periods of 24 months gave no further increase. At no stage was the growth definitely visible to the naked eye.

DISCUSSION

It is apparent from the somewhat conflicting reports of other authors and the work described in this paper that Johne's disease in sheep may be caused by one of two closely allied but different organisms. The first of these conforming to the classical *M. johnei* has long been recognised as a pathogen of cattle and sheep. The second organism, which as suggested by Taylor (1940) should be

distinguished as the ovine type, is the cause of an essentially similar disease and may only be differentiated from the first by its resistance to artificial cultivation. It may show a tendency to multiply slightly on artificial media after incubation for at least one year, but apart from one strain isolated by Dunkin and Balfour-Jones (1935), satisfactory growth has never been obtained. The very close relationship between this organism and *M. johnei* was demonstrated by McEwen (1939), who succeeded by its use in artificially infecting cattle which eventually developed symptoms and at autopsy, characteristic lesions of Johne's disease.

Paratuberculosis would appear to be a disease of comparatively low morbidity. Admittedly most of the cases examined came from hill flocks where the chances of infection must be considerably less than on enclosed land, but the statement is based on the fact that many clinical cases occur sporadically and only occasionally can a history of chronic emaciation among the stock be obtained. It has been shown that the disease is widespread in Scotland and there can be little doubt that its presence may often be unsuspected, particularly as many cases can be diagnosed only by a microscopic examination of stained films and that lesions may be present in the absence of clinical symptoms.

SUMMARY

Paratuberculosis or Johne's disease of sheep would appear to be widespread in Scotland.

The disease may be caused by either of two organisms, the classical type of *M. johnei* and a closely related organism which is only differentiated by its resistance to artificial cultivation and which may be termed *M. johnei* (ovine type).

REFERENCES

Dunkin, G. W. (1934). *Vet. Rec.*, **14,** 1551.
Dunkin, G. W., and Balfour-Jones, S. E. (1935). *J. comp. Path.*, **48,** 236.
Howarth, J. A. (1932). *J. Amer. vet. med. Ass.*, **81,** 383.
McEwen, A. D. (1939). *J. comp. Path.*, **52,** 69.
M'Fadyean, J. (1916). *Ibid.*, **29,** 62.
Minett, F. C. (1942). *J. Path. Bact.*, **54,** 209.
Stewart, J., McCallum, J., and Taylor, A. W. (1945). *J. comp. Path.*, **55,** 45.
Taylor, A. W. (1940). *Vet. J.*, **96,** 415.
Twort, F. W., and Ingram, J. L. Y. (1913). *Vet. Rec.*, **25,** 635.
Wilson, D. R. (1934). *Ibid.*, N.S. **14,** 1557.

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OBSERVATIONS ON THE BLOOD PICTURE OF JOHNE'S DISEASE IN SHEEP AND CATTLE WITH SPECIAL REFERENCE TO THE MAGNESIUM CONTENT OF THE BLOOD

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On routine examination of blood samples from cases of Johne's disease in sheep it was observed that both the calcium and magnesium contents of the blood were usually lower than the normal. Little has been recorded in the literature of variations in the mineral composition of the blood during infections or debilitating diseases. Stewart and Holman (1944) have shown that in the "pining" diseases known as "Cobalt-deficiency" and "Solway Pine," despite a considerable amount of emaciation there is little change in the mineral composition of the blood. Watchorn (1925) reported that in nearly all pathological conditions in man investigated by her, the serum magnesium was found to be increased. She also noted that there was no definite parallelism between calcium and magnesium. Various writers, Brockbank (1926), Halverson, Mohler and Bergeim (1917), amongst others, have shown that in tuberculosis the serum calcium values are on the average slightly lower than normal. They did not record magnesium values.

In the light of our preliminary observations, further cases of Johne's disease in sheep and cattle were examined. In all, blood samples were obtained from 19 sheep and nine cows. One of us (Taylor, 1945) has shown that Johne's disease in sheep may be caused by one of two closely related organisms and the majority of the sheep used in the present work was infected with the ovine type of *Myco. johnei*. On arrival at the Institute each ewe was diagnosed as a probable case of Johne's disease from clinical symptoms and a faeces examination for acid-alcohol fast organisms; confirmation was subsequently obtained on *post-mortem* examination. Of the cows, cases one to seven were diagnosed as Johne's disease as a result of clinical examination by veterinary practitioners; cases eight and nine were diagnosed from clinical symptoms and faeces examination. Blood samples from six cows (10 to 15) have been added to the experiment. These cows reacted to johnin but showed no clinical symptoms of the disease.

The results of the chemical analysis of the blood samples are recorded in the following table.

Examination of the data shows that the sugar, non-protein-nitrogen and chloride content of the blood was, with few exceptions, within the normal range. The one or two values outside the normal range may have been due to excessive emaciation, since a very debilitated animal has usually a low blood sugar and an abnormal blood chloride. In most cases the haemoglobin content of the blood was

ANALYSIS OF BLOOD SAMPLES—VALUES PER 100 ML. BLOOD

SHEEP

Animal	*Sugar* mg.	*Hb.* g.	*NaCl* mg.	*Ca* mg.	*Inorg. P.* mg.	*Total P.* mg.	*Mg.* mg.	*N.P.N.* mg.	*Amino N* mg.
1	45·6	11·0	550·0	7·60	4·32	10·28	1·62	39·2	5·6
2	34·5	6·4	590·0	8·26	5·50	9·62	1·39	33·7	4·8
3	23·8	5·4	580·0	6·26	4·90	9·25	1·49	28·6	4·9
4	55·8	6·3	580·0	8·53	2·76	8·00	2·08	28·6	5·8
5	40·6	7·3	550·0	7·86	3·84	7·94	2·77	—	6·9
6	38·6	9·3	510·0	7·00	5·44	10·80	2·32	28·2	6·8
7	29·8	9·9	480·0	8·10	4·14	9·00	1·60	28·6	7·4
8	47·2	8·1	520·0	8·60	4·32	9·30	1·60	43·5	7·2
9	68·5	5·9	560·0	7·80	3·83	6·54	2·07	33·2	5·5
10	57·2	8·0	560·0	7·70	4·32	8·32	1·67	21·8	5·7
11	—	—	—	8·40	—	—	1·84	—	—
12	75·9	9·2	580·0	10·60	—	—	2·08	—	—
13	52·1	10·7	580·0	8·80	5·06	10·60	2·13	30·0	4·2
14	41·3	8·9	580·0	8·00	5·90	9·20	1·95	35·3	4·7
15	52·9	5·6	530·0	6·80	4·19	7·40	2·12	37·7	3·5
16	44·4	5·5	550·0	8·00	3·86	6·10	2·50	28·7	3·5
17	45·8	9·1	540·0	8·50	3·86	7·70	2·30	35·9	3·7
18	54·3	10·7	510·0	7·30	4·52	9·80	2·06	32·4	4·0
19	58·1	7·7	540·0	8·40	4·52	8·30	1·98	38·5	4·2
Normal for Sheep	61·0 ±10·8	11·2 ±1·1	507·0 ±20·7	10·7 ±0·65	5·3 ±0·52	9·9 ±0·94	2·65 ±0·21	33·3 ±5·3	7·3 ±0·47

COWS

1	57·1	10·2	500·0	8·26	6·60	13·08	1·90	40·8	6·1
2	60·6	6·9	530·0	12·00	5·10	10·36	1·72	30·0	6·5
3	105·3	11·8	540·0	7·50	8·16	16·25	1·98	54·1	9·4
4	77·5	8·2	500·0	8·30	5·68	10·03	1·98	33·3	6·6
5	45·2	8·3	500·0	9·30	9·70	26·10	1·72	30·3	7·1
6	57·8	7·3	500·0	8·80	6·60	11·50	2·28	36·4	5·2
7	75·7	7·6	520·0	9·30	4·00	6·90	2·38	34·3	5·7
8	—	—	—	8·40	—	—	2·17	—	—
9	—	—	—	9·50	—	—	1·52	—	—
10	48·8	9·0	530·0	9·10	4·90	7·60	2·51	34·9	6·3
11	64·5	7·3	510·0	8·70	4·90	6·80	2·61	31·6	6·4
12	52·3	9·8	470·0	9·70	5·20	8·30	2·50	33·7	6·5
13	55·5	8·8	500·0	9·50	4·70	6·70	2·42	28·2	6·1
14	53·2	8·6	490·0	9·70	4·10	5·80	2·75	27·3	6·4
15	46·5	7·9	490·0	9·10	4·00	5·80	2·47	26·4	5·6
Normal for Cows	60·0 ±10·9	9·5 ±1·4	504·0 ±34·4	10·0 ±·72	4·24 ±0·87	7·7 ±1·20	2·98 ±0·28	38·9 ±7·0	4·6 ±0·20

very low, which again may show that there is a certain degree of anaemia brought about by emaciation similar to that seen in sheep heavily infested with helminths. The greatest variation from the normal is observed in the calcium and magnesium values. With the exception of No. 12, all the ewes had a low blood calcium and, with the exception of Nos. 5 and 16, a very low blood magnesium.

The variation from the normal was usually much greater in the case of magnesium than of calcium. The same was observed in Johne's disease in cows in which the blood magnesium was very much lower than normal, although the blood calcium was usually lower than normal or at the lowest range of normality. There is no parallelism between the low haemoglobin, calcium and magnesium contents of the blood since there may be an exceptionally low magnesium with a normal haemoglobin (sheep No. 1) or a normal magnesium with a low haemoglobin and calcium (sheep No. 5). In the cows, also, there was no strict correlation between the variations of the haemoglobin, calcium and magnesium values from the normal. It will be observed in cows Nos. 10 to 15 which reacted to johnin without showing clinical symptoms of Johne's disease, that, although the haemoglobin was slightly low in some cases, the blood calcium and blood magnesium were normal. Despite the general lowering of the calcium and magnesium, the phosphate content of the blood was usually normal although one or two cases had an exceptionally high blood phosphate.

DISCUSSION

As has been pointed out above, there is usually a very low haemoglobin content of the blood with occasionally a slightly low calcium content in severe debility such as that caused by heavy infestation of helminths. Recently Saifi and Vaughan (1944) have shown that infections also interfere with haemoglobin formation. There is no reference in the literature to any lowering of the magnesium content of the blood in infections or debility. In " cobalt-deficiency " and " Solway pine," debilitating conditions in lambs, Stewart and Holman (1944) have reported that the blood haemoglobin and the blood calcium are within the normal range whilst Stewart (unpublished observations) has shown the magnesium content of the blood in these two diseases to be 3·10 mg. $\pm$0·27 and 2·99 mg. $\pm$ 0·30 per 100 ml. respectively. It would appear, therefore, that the process of emaciation as such need not bring about a lowering of the magnesium content of the blood. In Johne's disease the cause of the condition may be due to some specific action of the bacteria. Future work may throw some light on this subject. From our present knowledge of blood pictures in various pathological conditions the lowering of the magnesium content of the blood in animals showing clinical symptoms of the disease may to some extent assist diagnosis since other useful aids such as examination of faeces for acid-alcohol fast organisms and testing with avian tuberculin or johnin have their limitations.

SUMMARY

It is shown that in sheep and cows exhibiting clinical symptoms of Johne's disease, the haemoglobin and calcium contents of the

blood may be low and that the magnesium content may be very low.

It is suggested that this lowering of the magnesium content of the blood may be a useful aid to the diagnosis of the condition.

REFERENCES

Brockbank, W. (1926). *Quart. J. Med.*, **20,** 431.
Halverson, J. O., Mohler, H. K., and Bergeim, O. (1917). *J. Amer. med. Assoc.*, **68,** 1309.
Saifi, M. F., and Vaughan, J. M. (1944). *J. Path. Bact.*, **56,** 189.
Stewart, J., and Holman, H. H. (1944). *J. comp. Path.*, **54,** 41.
Taylor, A. W. (1945). *Ibid.*, **55,** 51.
Watchorn, E. (1925). *Quart. J. Med.*, **18,** 288.

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OBSERVATIONS ON INFERTILITY IN A DAIRY HERD WITH SPECIAL REFERENCE TO *TRICHOMONAS FOETUS* INFECTION

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INTRODUCTION

This communication comprises a record of the clinical investigation of a herd of pedigree Friesian cattle in which the incidence of infertility was high, comprising when the writers were first consulted on September 16th, 1942, the following adult animals: *Cows, 61; heifers, 22; bulls, two. The cows were in the main young, for all except five were born during the years 1936 to 1939. The heifers were born between July, 1939, and November, 1940. The bulls were respectively five and four years old.

The tuberculin test revealed the incidence of tuberculosis to be high, 80 per cent. of the cows, 29 per cent. of the heifers and both bulls giving a positive bovine reaction. The general condition of the cows was good and they were high milk yielders. The majority of the heifers were in poor condition. It had been the practice on the farm for many years to vaccinate against *Brucella abortus* infection and all females had been treated as maiden heifers with the original live-culture vaccine prepared by the Ministry of Agriculture. The incidence of abortion had been negligible.

Difficulty had been experienced in getting cows and heifers in-calf for several years and the situation had become acute during the past year. Some returned to heat at frequent and fairly regular intervals but the majority showed oestrum only at long and irregular intervals. They were served whenever they were observed to be in heat.

A number of cows and heifers had had a white discharge from the vulva after service but none was known to have aborted a small foetus.† The two bulls (to be referred to as 1 and 2) had been on the farm since they were yearlings. They served vigorously, and no reduction in their eagerness had been noticed and no preputial discharge was seen. In many instances both bulls had been used on individual cows returning to heat. No new animals had been introduced for a number of years and no cows had been brought from other farms for service, but one of the bulls (No. 1) had been loaned to another farm for a period of about six months, and had returned on December 10th, 1941. The breeding records showed that with the exception of three, all the cows had calved normally during the period September, 1941, to August, 1942 (that is they

* Cows—animals which have borne one or more calves. Heifers—those which have been mated but have not yet calved.

† It should be noted that the animals had been at grass for the previous five months and it is possible that unobserved abortions had occurred.

conceived to services between January and November, 1941). This period is significant for *Trichomonas foetus* infection was subsequently found to exist. It strongly suggests that the disease was introduced by No. 1 bull at the end of 1941, although no confirmation of this was forthcoming.

PRELIMINARY FINDINGS

Cows.—Of the 61 cows, all were involved with the exception of six, which had recently calved. Reference to Tables II and III will indicate the calving dates before examination, the number of subsequent services and the bulls used. At the time of the first examination 13 were pregnant, but during the following six weeks four of these aborted foetuses 2·0 to 7·5 cm. long, and *T. foetus* was found in the foetal fluids and post-abortion discharges. Both bulls had been used on these abortion cases. The nine pregnant cows remaining carried their calves to term. Three cows were found to have pyometra : they had been served at times varying from four to eight months previously and had since been anoestrous. The uteri were distended and their walls tense ; in two the cornua were enlarged to an approximately equal degree and in the other the distension of the cornu on the side of the ovary containing the corpus luteum was distinctly greater. In all three cases one of the ovaries was larger and plumper than the other and as this state remained unchanged over a series of examinations, persistent corpus luteum was diagnosed. In two of them the uterine cervix was grossly hypertrophied with prolapse of the first fold through the external os, while in the other the cervix was small. The mucus occupying the cervix was moist and slippery and not tenacious as in pregnancy (Gould, 1939). A finger could be introduced into the cervical canal with ease and later no difficulty was experienced in passing a stout catheter through it into the uterus. There was an occasional vulval discharge of muco-flocculent material in which motile trichomonads could readily be found, but this discharge did not resemble the milk-like pus which was subsequently evacuated from the uterus. The tension in the uterus was greater than that of pregnancy and its walls were harder to the touch. No evidence of vaginitis was detected. The animals were in good condition and there was no sign of toxaemia resulting from the uterine infection.

T. foetus was isolated from 12 others. Clinically, they fell into two groups : (1) those in which no abnormality of the uterine cornua was detected and in which the parasite was found in the vaginal mucus of oestrum, and (2) those in which the cornua were appreciably enlarged and flaccid (approximately 7·0 to 11·0 cm. broad), and in which manipulation provoked a profuse vulval discharge. The nature of the discharge was constant—" glary," transparent mucus, similar to that which normally develops during oestrum, in which there were white floccules or streaks of a faint, nebulous opacity.

There remained 27 non-pregnant cows, served during the same period as the previous group, from which the parasite could not be isolated by microscopical examination. In these no positive anatomical abnormality to account for infertility could be found, but in two (284 and 326) ovaro-bursal adhesions were suspected. In a considerable number, hypertrophy and prolapse of the cervix was seen on speculum examination, but this was not considered to be the cause of infertility and subsequent events proved this view to be correct. In none was any abnormality of the vulva or vagina observed. A series of examinations indicated that in most the ovarian cyclical changes were normal, yet from the records of previous services and from their behaviour while under personal observation, it was clear that " silent heat " or suboestrum (Murray, 1943) was an important factor.

Heifers.—Twenty-two were involved. Their ages varied from two years to two years ten months. They had been served on one or more occasions by bull No. 1, commencing in April, 1942. Seven were pregnant and carried to term ; one had pyometra and *T. foetus* was isolated from three others. Of the 15 which were " empty," suboestrum was a feature in ten, and anoestrum was diagnosed in three.

TABLE I

SUMMARY OF FINDINGS IN COWS AND HEIFERS IN NOVEMBER, 1942

	Cows	*Heifers*	*Total*
Pregnant	9	7	16
Non-pregnant	52	15	67
Pyometra	3	1	4
Recent abortion	4	—	4
Others positive for *T. foetus*	12	3	15
Exposed to infection but parasite not found	27	11	38
Recent calvers not served since	6	—	6

Bulls.—No. 1.—This animal was five years old and a proved stock getter. During the period January to September, 1942, at least seven cows and heifers served by him only, developed trichomoniasis. During a similar period six animals mated by him on one or more occasions conceived and carried their calves to term.

Clinical examination of the bull revealed no abnormality of the genital organs and *T. foetus* was not found by microscopical examination in sheath washings. He was reluctant to use the artificial vagina and one thrust yielded 2 c.c. of semen. It was milk-white in appearance and *p*H 6·0 in reaction. Motility of the spermatozoa was excellent and they numbered 650,000 per c.mm. Some had detached heads and bent tails but the number of abnormal spermatozoa was not excessive. No use was made of this bull after the commencement of the enquiry and it was slaughtered four and a half months later. On *post-mortem* examination no trichomonads were found in any part of the genital tract. There were multiple small submucous haemorrhages on the glans penis, prepuce and urethra, and a small granuloma

(1·5 cm. broad) was present at the preputial reflection. The testes were normal but several thread-like adhesions occurred between the visceral and parietal peritoneum near the tails of the epididymis. Tuberculous lesions were found in the bronchial and right inguinal lymphatic glands.

No. 2.—Was four years old and a proved sire. Between February and September, 1942, six cows, served by this bull only, developed trichomoniasis, while three conceived and carried their calves to term. In this animal, also, clinical examination was negative.

The bull used the artificial vagina readily and semen examinations were made on four occasions between September, 1942, and January, 1943. The quantity of ejaculate varied from 3 to 5 c.c. Counts ranged from 880,000 to 2,064,000 per c.mm. The semen was densely opaque and yellow in colour. Motility was excellent and the number of abnormals low. In view of his pedigree and high reputation it was desirable that his semen should be used by artificial insemination and so he was first given three months' sexual rest. During this period the prepuce was irrigated on three occasions with a solution of Metaphen (Abbott) 0·02 per cent.* His semen was used on 17 cows ; five of these conceived with an I/C ratio of 1·4, and four carried their calves to term. The other aborted at 46 days and *T. foetus* was found in the foetal fluids. (It is highly improbable that this cow was infected before insemination for she was a recent calver which had not been served since.) Another cow " accidentally " served by him during this period, conceived and carried. Of the 12 in which conception failed, three were later found to be sterile and the remainder conceived to insemination by another bull.

The animal was destroyed four months later. On *post-mortem* examination no trichomonads and no gross lesions of tuberculosis were found. Submucous haemorrhages and peritoneal adhesions of the kinds described for bull No. 1 were also found in this case. The secretion of the seminal vesicles was yellow in colour but it was bacteriologically sterile.

In addition to the animals quoted as having been served by bulls 1 or 2 only, others were served during the same period by both. Of these two conceived, five became infected with *T. foetus*, and ten, while not conceiving, showed no signs of infection.

TRICHOMONAS FOETUS INFECTION

Course and Treatment

Of the factors responsible for infertility in this herd, *T. foetus* infection was believed to be of primary importance and treatment to combat it was immediately commenced. It was proposed at the outset to adopt the orthodox methods of uterine infusion using the technique introduced by Nielsen (1924), and employing the solutions of iodine and iodine with lactic acid recommended by Gould (1938), but as the work proceeded modifications were introduced. Natural service was to be withheld and artificial insemination, using the semen of clean bulls, instituted. In general, the plan was to

* " Metaphen." Makers' formula — 4-nitro-anhydro-hydroxy-mercuri-ortho-cresol. Contains w/w Hg. 0·125 per cent.

infuse the uterus in positive cases on three occasions at intervals of about a week, and in exposed but unconfirmed cases, once only. In order to reduce further loss of breeding time to a minimum, insemination was to be performed at the first or second subsequent heat according to the time elapsing between the last infusion and the onset of oestrum. Two new bulls were introduced. One (No. 3) was two years old and as far as was known had not been exposed to trichomonas infection, and the other (No. 4) was a yearling, which up to that time had done no work. Later the semen of No. 2 bull was also used.

Positive cases uncomplicated by pyometra or abortion.—Twelve cows and three heifers were concerned. Of the cows, two were treated with an aqueous solution of 0·1 per cent. iodine with 0·5 per cent. lactic acid; eight with 0·1 per cent. iodine alone; one with 0·01 per cent. mercuric chloride; and one with 0·02 per cent. Metaphen. 250 c.c. were used for each infusion, of which 200 c.c. were injected into the uterus and 50 c.c. into the cervical canal and vagina during withdrawal of the catheter. As initial efforts to pass the catheter through the uterine cervix in the heifers were unsuccessful, it was decided to make no further attempts, but instead to irrigate the vagina only, on one occasion using 0·1 per cent. iodine solution. The introduction of iodine both into the uterus and vagina, and vagina alone, was followed by severe straining, whereas no such straining followed the use of the solutions of mercuric chloride or Metaphen. In five of the cows motile trichomonads were found in the vaginal discharges 6 to 16 days after iodine treatment. They were not found after the use of mercuric chloride or Metaphen. Two of the animals in which trichomonads were found after iodine infusion of the uterus conceived to insemination without further treatment 21 and 40 days later.

Eleven of the cows and all the heifers subsequently conceived to artificial insemination with an I/C ratio of 2. This ratio would have been better had it not been for one cow, which required seven inseminations. The average time of conception after the termination of treatment was 66 days. This time lag was greatly increased by two cows and one heifer in which suboestrum supervened. All carried their calves to term except one heifer, which aborted at seven months. In this case the foetal stomach contents were positive for *C. pyogenes* but negative for *T. foetus* and *Br. abortus*. The remaining cow (338) was ultimately destroyed and *post-mortem* examination revealed unilateral ovaro-bursal adhesions with strangulation of the oviduct and hydrosalpinx.

Positive cases complicated by pyometra or abortion

Pyometra.—Three cows and one heifer. One of the cows was given 20 mgm. of stilboestrol dipropionate by intramuscular injection, but this did not appreciably influence her. Later the three cows were treated by passing a two-way catheter into the uterus. Much

of the pus was evacuated and the uterus was subsequently irrigated. After withdrawal of the catheter no further escape of pus occurred and 14 days later the uterine distension was as great as ever (Gilyard, 1942). At this point 40 mgm. of stilboestrol dipropionate was given intramuscularly and followed three days later by 6 c.c. of posterior pituitary extract. Within 48 hours of receiving the stilboestrol there was a profuse discharge of milk-like pus from the vulva which continued for about a week. The injection of pituitary extract caused a great increase in the discharge. In each case *T. foetus* was found in the pus and from two (296 and 341) *C. pyogenes* was isolated and from the third (333) an unidentified corynebacterium. Fourteen days later the cornua were contracted and their size was but little greater than normal. In all three animals oestrum occurred on the sixth day after the stilboestrol injection and at this time both ovaries in each were found to be small. A series of uterine infusions was then carried out using iodine solution. Oestrum recurred at irregular intervals. Two of the animals conceived (296 and 341) after periods of 130 and 163 days respectively, two and four inseminations being required. The calves were carried to term. The third cow (333) was destroyed three months later. This decision was influenced by the fact that she was fat, dry and of no particular value, and also by the desire to obtain her genital organs for *post-mortem* examination, for a series of rectal examinations had indicated that her ovaries had become " cystic " since the administration of stilboestrol.

Post-mortem examination, cow 333.

Cornua of normal width. Uterine secretion negative for *T. foetus* and bacteriologically sterile. Gross hypertrophy of the cervical folds. Histology of the endometrium (G. O. Davies)—" an infiltration of the m.m. with lymphocytes, endothelial cells and a few leucocytes. Also what appears to be a marked hyperplasia of the blood vessels. The vessels are more numerous and better developed than normal." Right ovary: dimensions —4·8 × 3·3 × 3·2 cm. A central cyst of 2·0 cm., and a second of 1·6 cm. No lutein tissue in their walls. Numerous follicles up to 1·0 cm. Left ovary: 4·6 × 3·0 × 2·5 cm. Two cysts of 2·6 cm. and numerous follicles up to 1·0 cm. No lutein tissue. Several corpora albicantes but no coloured remnants.

Oviducts, fimbriae and bursae—normal.

No local treatment was applied to the heifer and it serves as a control in this respect. The uterus was evacuated by the action of stilboestrol dipropionate 25 mgm. and pituitary extract 4 c.c. The pus was positive for *T. foetus* and *C. pyogenes*. Oestrum was first observed 43 days later, but insemination failed. A period of suboestrum supervened but no treatment was applied and heat recurred after a further 68 days. The heifer was then mated (" accidentally ") and conceived and carried to term. (See protocol of bull No. 4.)

Abortion.—Six cows. Four aborted after service by bulls 1 and/or 2, one after insemination by No. 2, and in one the accident

was inadvertently provoked 86 days after service by No. 2. *T. foetus* was found in each. All were treated by intra-uterine infusion; two with iodine and the remainder with Metaphen. One (330) was later destroyed and *post-mortem* examination revealed ovaro-bursal adhesions and tuberculous salpingitis, and the remainder conceived to insemination with an I/C ratio of 2. The average time after treatment at which conception occurred was 137 days, but two were the subjects of suboestrum. They carried their calves to term.

Cows and heifers exposed to T. foetus *infection from which the parasite was not isolated*

This group comprised 27 cows and eleven heifers. Twenty-two of the cows were treated by intra-uterine infusion of iodine and one by Metaphen; four received no local treatment. Of the heifers, six received a single vaginal douche with iodine and five no treatment. The cows, except four, and all the heifers subsequently conceived to insemination with an I/C ratio of 1·97. The exceptions were later destroyed. In two of them (297 and 305) no cause for the infertility was found; in the third (284) there was miliary tuberculosis of the endometrium and bilateral ovaro-bursal adhesions, and in the fourth (326) unilateral ovarian adhesions.

The average time which elapsed before conception occurred in those animals in which oestrous cycles were normal was 45 days. (This calculation is made from the time of the last uterine or vaginal infusion or, in those which received no treatment, from the date of first examination.) In eight, however, suboestrum developed and in these the average delay was 174 days. In two heifers in which anoestrum was present, conception occurred after an average lapse of 205 days. It is of interest to note that one cow conceived as early as eight days after iodine treatment of the uterus and four others did so within 20 days. All this group carried their calves to term or were disposed of shortly before they were due to calve, with the exception of one (335) which aborted at 228 days. The foetus was putrid when expelled but *T. foetus* could not be found.

TECHNIQUE OF ARTIFICIAL INSEMINATION

The methods adopted for the collection and injection of semen were orthodox and require no description. Fresh, undiluted semen collected immediately before use was employed. As a rule the cow or heifer to be inseminated was used to stand for the bull while collecting semen. Those first seen in heat in the morning were inseminated late the same afternoon, while those which developed oestrum in the evening were dealt with the following morning. A point to which attention should be drawn is " accidental " mating during the collection of semen, for it may be a serious matter in a Trichomonas infected herd. In a series of about 250 collections made by one of the writers (G. H. A.) in this and other herds, it

has happened three times. It is most likely to occur when using a cow in which heat is passing off. As the bull mounts she suddenly "swings round"; the bull quickly follows and is able to effect service before the operator reaches the new position. No difficulty of bulls refusing to use the artificial vagina has been experienced, but with the exception of bull No. 2, all the animals used have been young ones.

Bull No. 3.—This animal's semen was used during October and November, 1942, in 12 cases. Seven conceived to a single insemination. Of the remainder, four subsequently conceived to other bulls and one was sterile. Semen was examined on three occasions. Its appearance was dense, milky-white; motility was excellent; *p*H varied from 6·5 to 8, and counts ranged from 556,000 to 1,096,000 per c.mm.

Bull No. 4.—Was born on 22.9.41 and purchased as a calf. Its utilisation for artificial insemination was commenced on 20.10.42, and up to the time of compiling this record (7.2.44) 113 semen collections had been made. At all times he used the artificial vagina readily and vigorously. Of the cows and heifers under review, 66 conceptions have resulted from the use of his semen with an I/C ratio of 1·6. Semen was examined on 29 occasions. It varied from a thin, whey-like liquid to a dense, creamy one in appearance. Quantities ranged from 0·75 to 7·0 c.c. per ejaculate. Motility varied but generally was good. Counts were from 332,000 to 2,132,000 per c.mm.

On 22.4.43 he served heifer 330H, which three and a half months previously had been the subject of pyometra (*T. foetus* and *C. pyogenes*). The heifer conceived and the following treatment was applied to the bull: The lining of the artificial vagina was smeared with "Dettol" ointment and the bull served into it three times. The preputial orifice and the anterior part of the sheath was anointed by hand. Since that time his semen has been used for insemination on many occasions but no sign of Trichomonas infection has been seen.

HYPERTROPHY OF THE UTERINE CERVIX

This condition was seen on speculum examination in many of the animals concerned in this review. Three of these (284, 326 and 338) were later found to be sterile from causes other than the cervical deformity, and the remainder conceived to insemination (ratio 2·07). A slight degree of protrusion of the first fold was seen in several heifers, and one of them was found to have a gross prolapse when reinseminated after calving.

ABNORMALITIES IN THE OESTROUS CYCLE

From the outset it was obvious that great loss of breeding time was due to irregularity in the frequency of observable heat in non-pregnant animals. Reference to the service dates prior to the commencement of the investigation showed that often there were periods

of three or four months between services and in one cow, in which rectal examination revealed no abnormality, heat had not been seen for seven months. A common history was that an animal came into season once or perhaps twice after calving, was served but failed to conceive, and then showed no sign of heat for several months. It was not certain that some had not aborted unobserved at grass, but that explanation does not account for all, for during the first six months the herd was under the writers' observation the animals were housed and it is very improbable that any unseen abortions occurred; nevertheless the absence of heat persisted in many. The cowman was one of experience who took a keen interest in his cattle and failure to observe heat could not be ascribed to inefficiency. In order to reduce error to a minimum, one of the bulls was walked through the cowshed each evening and there is no doubt that by this practice heat was detected in many cows which otherwise would have been missed. Any animal which showed the slightest interest in the bull was introduced to him the following morning. If she "stood," artificial insemination was performed. If she refused, but rectal examination revealed a turgid uterus, the absence of an appreciable corpus luteum in either of the ovaries, and a copious quantity of mucus in the vagina, insemination was also carried out. During the 18 months the herd was under observation up to the time of compiling this report, out of a total of 98 animals, absence of heat for two months or more was encountered in 41. In addition to the well recognised conditions of suboestrum in which the processes of follicle development, ovulation and corpus luteum formation are normal, and anoestrum in which the ovaries remain small and functionless, a third condition of "cystic" ovaries was observed associated with absence of heat.* In these cases a series of rectal examinations of the ovaries revealed no sign of a corpus luteum but several abnormally large follicles in one or both, which remained unchanged. Pinching sometimes ruptured a follicle (but this did not provoke heat) but more often they were resistant to pressure. The condition persisted for several months and then resolved spontaneously. The following is a record of an animal which was the subject of this condition in which *post-mortem* evidence was available:—

349. Born 5.7.38. Calved to service 26.4.41 and 4.4.42. *T. foetus* not found. Calved to artificial insemination 28.7.43. Oestrum occurred on 21.8.43, 27.9.43 and 9.11.43. From this time until the animal was slaughtered in May, 1944, there were no signs of heat. The ovaries were examined on many occasions and were found to be abnormally large due to excessive follicle development.

Post-mortem findings: Right ovary $4{\cdot}6 \times 3{\cdot}2 \times 3{\cdot}2$ cm. A multilocular cyst of 2·6 cm. with no sign of lutein tissue in its walls. A second follicle

* In the cow cystic ovaries are generally regarded as being associated with an excess of heat. Although this is often the case the object of this communication is to indicate that they may be associated with absence of heat.

of 0·8 cm. with some recent luteinisation of its wall. Several adventitious " strings " attached to the surface of the ovary but the fimbria, bursa and oviduct normal. Left ovary 3·8 × 2·6 × 3·0 cm. Three cysts of 2·5 cm. In the wall of one of them were localised areas of orange-coloured lutein tissue. No other abnormalities were found in the genital organs.

In one animal a gross cyst-adenoma of the ovary was associated with anoestrum for a period of eight months after which nymphomania developed.

Treatment

Stilboestrol dipropionate.—Ten cases of suboestrum were treated by the intramuscular injection of this agent in doses ranging from 15 to 25 mg. In five, visible heat developed in from one to six days. None of them conceived to insemination at the provoked heat, but in one of the animals in which heat was not detected, insemination on the third day after injection caused conception. In three of the induced heat cases, recurrent cycles developed and in the other two suboestrum again supervened. It will be recalled that in the three pyometra cases in cows, a dose of 40 mg. provoked heat in each on the sixth day, and was followed by recurrent but irregular visible cycles. The ovaries of one (333) had not returned to normality when the animal was destroyed three months later.

A dose of 30 mg. was given to each of two cows in which recurrent cycles were normal, three days and 69 hours respectively, before slaughter. No change in the ovaries ascribable to the agent could be detected at autopsy.

Expression of the corpus luteum.—The operation was performed in 27 cases of suboestrum and in none did the animal suffer appreciable harm therefrom. In 19 (70 per cent.) visible heat occurred in a period of one to five days ; 17 were inseminated at the provoked oestrum ; ten conceived and in two the result is not known at the time of writing. In those which failed to conceive, recurrent cycles developed. One cow which did not show heat was inseminated three days after operation, and conceived.

Pregnant mare's serum.—During 1943, P.M.S. was used in seven cases of anoestrum ; three heifers and three first calvers ; 1,500 units was used on five occasions. Visible heat occurred in two only, in each on the fourth day after injection. Insemination was unsuccessful and anoestrum again supervened ; 3,000 units were also used five times. In one case only did visible heat follow —on the eleventh day, and insemination failed. In three cases the ovaries became abnormally large as the result of the agent and *super* follicle development (the development of an excessive number of large follicles without ovulation) was diagnosed. Natural heat occurred after variable periods ranging from 24 to 69 days. In one animal a profuse, opaque vulval discharge developed : from it, coliform organisms only were isolated in culture.

In 1944, four anoestrus heifers which were in poor condition

were given 2,250 units. On each of the succeeding nine days they were allowed to run in a yard with a bull, but on no occasion would any of them stand. Five days after injection follicles could be detected in the ovaries and the uterine cornua were turgid. On the ninth day it was evident that *super* follicle development had occurred, for seven of the eight ovaries concerned varied in size from a walnut to a hen's egg, and follicles up to an approximate diameter of 2·5 cm. could be palpated. In each case a yellow, opaque vulval discharge developed and in one the discharge could be seen exuding from the uterine cervix on speculum examination. Cultural examination revealed no significant organisms. On the sixteenth day the ovaries were unchanged and each was submitted to vigorous squeezing but no indication of expression of a corpus luteum occurred. Thirty days after injection the ovaries had reverted to the anoestrous state.

SUMMARY

The clinical course of an outbreak of infertility in a herd of Friesian cattle is described.

The chief causes of the condition were: (*a*) *Trichomonas foetus* infection with failure to conceive, abortion and pyometra. (*b*) Endocrine dysfunction associated with absence of heat and classified, according to the ovarian findings on rectal examination, as suboestrum, anoestrum and cystic ovaries.

Of 77 females exposed to *T. foetus* infection by service, 16 (21 per cent.) conceived and carried to term and no evidence of the disease was seen; 23 (30 per cent.) became infected and of these four aborted and four others developed pyometra; 38 (49 per cent.) failed to conceive but the parasite was not isolated from them.

Trichomonads were not recovered from the bulls used during the period of spread of infection, either during life or *post mortem*.

Cows and heifers exposed to *T. foetus* infection were treated in several ways. In most, uterine infusion was adopted and a variety of antiseptic agents were used. In some, vaginal douching only was employed while in a few no local treatment was applied. The great majority of the animals subsequently conceived. To combat the spread of Trichomonas infection, natural service was replaced by artificial insemination, using, in the majority of instances, the semen of clean bulls.

The semen of a "carrier" bull was used by artificial insemination in 17 animals, in one of which infection resulted.

Pyometra cases failed to respond to treatment by syphonage and irrigation of the uterus, but the injection of stilboestrol dipropionate followed by posterior pituitary extract was followed by evacuation of the pus. Three out of four cases subsequently conceived and carried their calves.

In the treatment of suboestrum, expression of the corpus luteum was found to be the most satisfactory method. Anoestrum and cystic

TABLE II

SUMMARY OF COWS AND HEIFERS POSITIVE FOR *T. Foetus* INFECTION

No.	Date of Last Calving	No. of Services since	Bulls used	Nature of Local Treatment	Subsequent History: No. of Insem-inations	Bulls used	Result	Calving Date	Gesta-tion Days	Sex of Calf	Remarks
					A. Pyometra cases						
296	20. 4.42	1	2	Uterus—Iodine	2	2 & 4	+	17. 1.44	282	F.	
341	15. 1.42	1	1	" "	4	4	+				Sold as "springer."
333	12.11.41	2	1 & 2	" "	1	2	—				Destroyed—see protocol.
330H	Heifer	2	1	None	2	4	+	31. 1.44	284	F.	
					B. Abortion Cases						
303	25. 2.42	Uncertain		Uterus—Iodine	1	4	+	9. 9.43	278	M.	
312	10.12.41	1	2	" "	5	4	+	31. 3.44	283	M.	
315	11.11.42	1a	2	" Metaphen	1	4	+	7. 2.44	275	M. & F	
320	31. 7.41	4+	1 & 2	" "	1	3	—				Destroyed.
362	13. 7.42	2	2 & 1	" "	1	4	+	4. 2.44	277	F.	
373	11. 8.42	1	2	" "	2	4	+	30. 1.44	281	F.	
					a—Insemination						
					C. Uncomplicated (non-pregnant) cases						
306	28. 3.42	1	2	Uterus—Iodine, lactic ac.	1	4	+	8. 9.43	282	F.	
313	29. 6.42	1	2	" " "	1	4	+	20. 9.43	285	M.	
316	7. 9.41	6+	1 & 2	" $HgCl_2$	2	4 & 2	+	19. 9.43	276	F.	
338	31.12.41	2	1 & 2	" Iodine	1	4	—				Destroyed.
346	27. 3.42	1	2	" "	2	4	+				Sold as "springer."
358	5. 3.42	1	?	" "	7	2 & 4	+				Sold as "springer."
359	29. 3.42	1	1	" "	1	3	+	3. 6.43	221	M.	Premature calf, lived.
361	9. 4.42	1	2	" "	1	4	+	1. 8.43	274	F.	
363	19. 6.42	1	?	" "	3	4	+	2.12.43	279	M.	
365	29. 6.42	1	1	" "	2	3 & 2	+	20. 9.43	280	M.	
392	Heifer	2	1	Vagina "	2	4	+	19. 8.43	281	M.	
395	"	1	1	" "	2	4	+	6.10.43	279	F.	
287H	"	1	1	" "	2	3 & 4	+	25.12.43			Abortion at seven months.

No.	Date of Last Calving	No. of Services since	Bulls used	Nature of Local Treatment	Subsequent History: No. of Insem-inations	Bulls used	Result	Calving Date	Gesta-tion Days	Sex of Calf	Remarks
270	31. 5.42	3	1 & 2	Uterus—Iodine	2	3 & 4	+	16. 8.43	277	F.	
284	29.10.40	2+	1 & 2	" "	2	4	—				
285	13. 2.42	5	1 & 2	" "	2	2 & 4	+	21.12.43	281	F.	Destroyed.
289	22. 2.42	1	2	" "	1	4	+				
293	17.10.41	2	2	None	3	2 & 3	+	26. 7.43	283	M.	Sold as "springer."
297	1. 2.41	3	1 & 2	"	1	2	—				
302	20. 4.42	1	2	Uterus—Iodine	2	2 & 4	+	12.12.43	277	F.	Destroyed.
305	14. 3.42	2	1 & 2	" "	9	4	—				
307	10. 3.42	2	1 & 2	" "	1	4	+	28. 7.43	273	F.	Destroyed.
318	19. 7.42	1	2	" "	3	2 & 4	+	4. 3.44	287	F.	
319	4. 6.42	1	2	" "	5	4	+	4. 4.44	286	F.	
326	22. 1.42	9	1 & 2	" "	6	2 & 4	—				Destroyed.
330	7.12.41	2	1	" "	1	3	+				Sold as "springer."
335	29.11.41	3	1 & 2	" Metaphen	2	2 & 4	+				Abortion at seven months.
336	26.12.41	2	1	" Iodine	1	4	+				Sold as "springer."
343	24. 2.42	1+	2	" "	5	3, 2 & 4	+	19. 2.44	282	M.	
344	4. 3.42	3	1 & 2	" "	1	4	+	1. 8.43	292	M.	
347	28. 5.42	2	2	" "	1	4	+	19. 7.43	281	F.	
348	3. 3.42	2	2	" "	1	4	+	12. 8.43	283	M.	
349	4. 4.42	1	2	" "	1	4	+	28. 7.43	268	F.	
352	27. 1.41	5	1 & 2	" "	5	4	+	9. 3.44	284	M.	
353	20. 9.41	3	1 & 2	" "	1	4	+	27. 8.43	280	M.	
357	13. 2.42	1	2	" "	2	4	+	26. 2.44	274	M.	
360	1. 4.42	1	1	None	1	3	+	1. 8.43	280	M.	
366	4. 7.42	1	?	Uterus—Iodine	3	2 & 4	+	27.12.43	274	M.	
370	30. 7.42	1	2	None	1	4	+	24.12.43	281	M.	
M.M.	Uncertain	1	1	Uterus—Iodine	2	4 & 2	+	13.10.43	282	F.	
393	Heifer	3	1	Vagina "	1	3	+	20. 8.43	274	F.	
394	"	2	1	None	1	4	+	14. 8.43	267	F.	
396	"	2	1	"	2	4 & 2	+	19.10.43	296	F.	
398	"	3	1 & 2	"	1	3	+	2.11.43	283	M.	
399	"	1	1	Vagina—Iodine	4	2, 3 & 4	+	14.11.43	277	F.	
324H	"	1	1	None	1	4	+				
391	"	1	2	Vagina—Iodine	1	3	+	18. 8.43	275	M.	Sold as "springer."
331H	"	2	1	" "	4	4	+	7. 2.44	278	M.	
389	"	2	1	None	1	4	+	23. 7.43		M.	
285H	"	1	1	Vagina—Iodine	1	5*	+	11. 3.44	274	M.	
MMH	"	1	1	" "	4	4 & 2	+	3.10.43	277	M.	

* A young bull not previously used

ovaries did not respond to treatment but natural recoveries occurred.

Eight cows were destroyed as sterile. In two no abnormality to account for continued infertility was found, in two there was tuberculosis of the genital tract and in three ovaro-bursal adhesions. In one there was a gross cyst-adenoma of the right ovary.

Hypertrophy of the uterine cervix and prolapse of the first fold through the external os was present in many of the cows. It was not found to be a cause of infertility.

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REFERENCES

Gilyard, A. T., and Gilyard, R. (1942). *J. Amer. vet. med. Ass.*, **101**, 276.
Gould, G. N. (1939). *Vet. Rec.*, **51**, 1463.
Murray, J. G. (1943). *Ibid.*, **55**, 323.
Nielsen, F. (1924). *Ibid.*, **4**, 835.

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THE COAGULASE TEST FOR STAPHYLOCOCCI

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INTRODUCTION

The coagulase tube test for the rapid identification of pathogenic staphylococci has long been employed in the examination of strains of human origin and is generally accepted as being of great value.

More recently, a modification of the test has been used as a rapid slide test and the results obtained have compared favourably with those obtained by the tube test (Cadness-Graves *et al.*, 1943; Berger, 1943; Birch-Hirshfield, 1944). Some variation occurs in the technique. For the tube test that used by Fisk (1940) seems to be the most commonly employed. In this, five drops of an overnight broth culture are added to 0·5 c.c. of a 1:10 dilution of human plasma in saline. The tubes are incubated for three hours at 37° C. and then left on the bench at room temperature overnight. In positive tubes the plasma is coagulated. For the slide test, the procedure recommended by Cadness-Graves *et al.* is to emulsify a suspect colony in a drop of water and to add a drop of undiluted human plasma. In a positive reaction large and irregular clumps appear as soon as the plasma comes into contact with the organisms. Berger found that all strains examined by him which were coagulase-positive by the tube test were coagulated by plasma on a slide, but that a few coagulase-negative strains were agglutinated. There was, however, a marked difference in the reaction obtained with the latter strains for, at first, on the addition of plasma an even suspension resulted and it was only after a minute or two that small and irregular clumps appeared. He attributed this reaction to natural agglutinins in the plasma whereas the true coagulase-positive reaction is not regarded as agglutination.

On the hypothesis that the reaction in both tests is due to coagulase on the surface of pathogenic staphylococci which coagulates fibrinogen on coming into contact with it, Berger investigated the value of fibrinogen for the slide test. This he prepared in crude solution by adding a saturated solution of sodium chloride to an equal volume of citrated human plasma and keeping the mixture in an ice chest for four hours. The precipitated fibrinogen was separated by centrifugation and redissolved in an amount of distilled water equal to that of the original volume of the plasma. Using this crude fibrinogen, Berger carried out slide coagulase tests with the strains previously used and found that coagulase-positive strains were clumped by the fibrinogen but that no coagulase-negative strains were agglutinated, the reaction attributable to natural agglutinins in the blood being eliminated. He concluded, therefore, that untreated plasma should not be used but that by testing suspect colonies for coagulability with fibrinogen a rapid determination of the pathogenicity of the strain was possible. Further, the test was just as reliable as the tube test. A point noted by Much (1908) and confirmed by other observers is that an occasional strain of staphylococcus is encountered which will not form an homogeneous suspension with water and in which a slide test is therefore impracticable.

Fairbrother (1940) concluded from his observations with human strains of staphylococci that the genus Staphylococcus should be subdivided into coagulase-positive strains, designated *Staphylococcus pyogenes* and coagulase-negative strains designated *Staph. saprophyticus.*

Staphylococci are amongst the most commonly isolated organisms from pathological material of animal origin but their significance in the pathological process is often a matter of doubt. Haemolytic activity and pigment production have been the most commonly accepted criteria as to the possible pathogenicity of animal strains.

A. BOVINE STAPHYLOCOCCI

Experimental

The initial experiments were confined to 124 strains of staphylococci isolated from the bovine udder. Some were from acute cases of mastitis, others from udders showing, by virtue of their cellular content, evidence of udder disturbance, while the majority were isolated from milk samples submitted for routine examination for evidence of mastitis. Strains producing filtrable haemolysin for sheep red blood cells were classed as pathogenic and those not producing filtrable haemolysin as non-pathogenic. All samples were submitted to the tube test following the technique of Fisk but using *bovine* plasma and to the slide test following the method described by Cadness-Graves *et al.* using *bovine* plasma, fibrinogen and serum. The latter was included as an additional check on agglutinable strains.

Results

Unfortunately, accurate results relative to this phase of the work cannot be given as the data have been destroyed by enemy action,* but it is possible to give some general impressions of the experiments. The slide test proved eminently successful and was found to be a quick, easy and reliable method to decide whether a given strain of staphylococcus was pathogenic or not. Of the pathogenic strains, all were coagulated by plasma and fibrinogen, whereas no strains classed as non-pathogenic were so coagulated. Two strains were encountered which would not form an homogeneous suspension with water and which could not, therefore, be submitted to the slide test. A very occasional strain was also met with which gave an agglutination with plasma and serum. The reaction in these cases was much slower in developing than in a true coagulase reaction and the resultant clumps were much smaller, so that no difficulty was experienced in differentiating between an agglutination reaction and a coagulase reaction. Nevertheless, we concluded that to eliminate the possibility of error, it was preferable to use crude fibrinogen rather than plasma for the slide test, although the latter was sufficiently good for routine diagnostic purposes.

* The author's accepted text was destroyed while in the press.—EDITORS.

Difficulty was at first experienced with the tube test and it was noted that great care is necessary in its successful employment. Final results were, however, in general agreement although occasional strains were met with, the reactions of which were difficult to assess. Our impressions appear to bear out the findings of Gillespie (1943), who has pointed out the difficulties of the tube test and the errors that may arise in unskilled hands. Comparing the tests we found the slide test to be superior to the tube test and considered it much more satisfactory for general application.

It is interesting to note that in this series of strains which we examined all those classified as pathogenic produced on 10 per cent. sheep blood agar either the typical double zone of haemolysis, *i.e.*, a clear zone surrounded by a wider darkened band or were surrounded by a darkened band only. None only produced the clear zone of haemolysis which is characteristic of pathogenic human strains. Most of the non-pathogenic strains produced no haemolysis on sheep blood agar although quite a few did produce a narrow clear zone only. During the whole of this work, while endeavouring to classify the strains according to pigment production, we experienced difficulty and inability to decide as to the colour of the pigment produced by various strains under observation. We found that nine strains classified as *aureus* were coagulase-negative and did not produce filtrable haemolysin. As there has been a tendency to accept *Staph. aureus* as pathogenic, this finding gives support to objections against dividing the genus on the basis of pigment production.

B. STAPHYLOCOCCI FROM VARIOUS SOURCES

Encouraged by the results obtained with the udder strains, we proceeded to investigate the coagulase production of staphylococci isolated from various sources upon plasmas which were easily accessible to us in the laboratory. Studies of this nature have previously been made by several workers using the tube test only but it appears that the slide test has not been similarly investigated. In view of this fact, and as we had obtained such satisfactory results with the slide test, we decided to confine our attentions to this test only. Cruikshank (1937) had shown that coagulase is not produced solely by strains of human origin, for six animal strains examined by him were coagulase-positive on tube test using presumably human plasma. Further, he showed that the plasmas of the horse, ox, sheep and guinea-pig, diluted 1:10 with saline, were coagulable by the same test, but those of the horse and guinea-pig were clotted more quickly than those of the ox and sheep. Wilson Smith and Hale (1944), however, using human strains of staphylococci, found guinea-pig, fowl and mouse plasmas to be uncoagulable by the tube test. This they considered to be due to a total absence of an activator substance in the two latter plasmas and a deficiency of this substance in

guinea-pig plasma which was found to clot very slowly at 20° C. instead of 37° C., and only when large doses of coagulase were used. The addition of human or rabbit testis extract or blood sera, which contains activator substance, was found to render these three plasmas fully coagulable. Cowan (1938) has also examined staphylococci of animal origin by the tube test but not with a variety of plasmas.

Experimental

Strains of staphylococci of food (9), human (33), ox (31), sheep (17), horse (2), dog (12), rabbit (1), and goose (5) origin were tested with human, ox, sheep, horse, dog, swine, guinea-pig, mouse, rabbit and cat plasmas. Serum and fibrinogen were only used when a check on the results obtained with a given strain seemed desirable.

The vast majority of the pathogenic strains were obtained from active lesions or infections and were only accepted as such if they were capable of producing filtrable haemolysin for sheep red blood cells. A notable exception occurred in the case of five strains isolated from outbreaks of staphylococcal arthritis in goslings. These strains were not haemolytic on sheep blood agar neither did they produce filtrable haemolysin, but their isolation from as many as seven sites in the fresh cadaver in each case, together with their other characteristics, compelled the view that they were actively pathogenic. An attempt was also made to classify strains according to pigment production. Complete agreement was only obtained with 74 out of 87 strains after cultivation for 24 hours at 37° C. and keeping at room temperature for a further period.

Results.—(These are summarised in Table I.)

TABLE I

COAGULASE ACTION OF PATHOGENIC STAPHYLOCOCCI OF VARIED ORIGIN ON PLASMAS OF DIFFERENT SPECIES OF ANIMALS

	Origin of Strains															
	Food		*Human*		*Ox*		*Sheep*		*Dog*		*Horse*		*Rabbit*		*Goose*	
Plasma	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—
Human	6	0	19	0	20	0	14	0	8	0	1	0	1	0	5	0
Bovine	6	0	19	0	20	0	14	0	8	0	1	0	1	0	5	0
Mouse	6	0	19	0	20	0	14	0	8	0	1	0	1	0	5	0
Dog	6	0	18	1	20	0	14	0	8	0	1	0	1	0	5	0
Swine	6	0	19	0	17	3	14	0	8	0	1	0	1	0	5	0
Rabbit	6	0	19	0	20	0	14	0	8	0	1	0	1	0	5	0
Cat	6	0	19	0	20	0	14	0	8	0	1	0	1	0	5	0
Horse	3	3	8	11	12	8			8	0	1	0	1	0	5	0
Sheep	0	6	0	19	10	10	0	14	0	8	0	1	0	1	0	5
G. Pig	0	6	0	19	9	11	0	14	8	0	0	1	0	1	0	5

Food Strains.—Of the nine strains examined, six which were classed as pathogenic all coagulated human, ox, mouse, dog, pig,

rabbit and cat plasmas but only three coagulated horse plasma and none coagulated the plasmas of the sheep and guinea-pig. None of the three non-pathogenic strains coagulated any of the plasmas.

Human Strains.—Of the 33 strains examined, 19 which were classified as pathogenic, likewise all coagulated human, ox, mouse, pig, rabbit and cat plasmas ; one did not coagulate dog plasma, while only 8 coagulated horse plasma ; none coagulated the plasma of the sheep or guinea-pig. None of the remaining 14 strains, classified as non-pathogenic, coagulated any of the plasmas.

Bovine strains.—From the 124 strains previously examined 31 were selected for the present work and of the 20 classified as pathogenic, all again coagulated human, ox, mouse, dog, rabbit, and cat plasmas ; three, however, failed to coagulate swine plasma and as with the food and human strains, a high proportion, namely, eight, failed to coagulate horse plasma ; in contrast, a strikingly large proportion, namely ten and nine, respectively, had a strong positive action on the sheep and guinea-pig. None of the eleven non-pathogenic strains coagulated any of the plasmas.

Ovine strains.—Of the 17 strains available for test, 15 were classed as pathogenic, and one of these would not form a homogeneous suspension in water and so could not be submitted to the slide test. The remaining 14 coagulated all plasmas except those of the sheep and guinea-pig. It is noteworthy, therefore, that all the pathogenic strains isolated from sheep did not coagulate sheep plasma, although some bovine strains did so. The two non-pathogenic strains were coagulase-negative throughout. Reactions with horse plasma were very weak and are not included in the table.

Canine strains.—Twelve were available for test, and of the eight classed as pathogenic, all coagulated human, ox, pig, rabbit, dog, cat, and guinea-pig plasmas and gave a weak reaction with horse plasma. None coagulated sheep plasma. The four non-pathogenic strains were coagulase-negative throughout.

Equine strains.—Two strains only were tested, namely, one which was pathogenic and one non-pathogenic. The pathogenic strain coagulated the plasmas of all species except the guinea-pig and sheep. The non-pathogenic strain was coagulase-negative throughout.

Rabbit strain.—One strain only was tested which was pathogenic and coagulated the plasmas of all species except the guinea-pig and sheep.

Avian strains.—The five strains mentioned above as having been isolated from outbreaks of staphylococcal arthritis in goslings coagulated all plasmas except those of the sheep and guinea-pig. These strains were non-haemolytic on sheep blood agar and produced no demonstrable filtrable haemolysin on test with sheep and rabbit red blood cells. On rabbit and guinea-pig blood agar they produced a narrow but clear zone of haemolysis. No haemolysis was produced on a blood agar plate containing 10 per cent. hen's blood.

DISCUSSION

These results suggest that coagulase is produced only by pathogenic staphylococci and that the plasma of the human, ox, mouse, rabbit, or cat species can be used to identify pathogenic strains. Very occasional strains may not coagulate the plasmas of the pig and dog. The intensity of the reaction obtained using horse plasma was very weak throughout and great difficulty was often experienced in deciding whether or not a reaction occurred. Hence, it was concluded that horse plasma is unsuitable for use in tests. The hypothetical enzyme or activator present in plasma which gives rise to coagulation in the presence of pathogenic staphylococci may, therefore, occur in horse plasma in a concentration that is too low for its employment in critical differentiation tests. On the basis that the slide and tube tests are an illustration of the same phenomenon, the results obtained with guinea-pig and sheep plasma render it difficult to explain the coagulant action on the theory of Wilson Smith and Hale or upon any other suggested hypothesis; about half the pathogenic bovine strains coagulated the plasmas of both these species and all the pathogenic canine strains coagulated guinea-pig plasma. The reaction in all these cases where it was positive was a strong one and unlike the weak positive reaction seen with horse plasma. All the other strains examined had no effect on guinea-pig and sheep plasmas. As a result of the work of Wilson Smith and Hale, human serum was added to these plasmas. No effect on coagulability by the slide test was observed.

Some strains of pathogenic staphylococci give a much more intense reaction than others. This would suggest that some strains produce more coagulase than others, and it is possible that a correlation may exist between coagulase production and pathogenicity. On the other hand, the fact that pathogenic strains of ovine origin did not coagulate sheep plasma does not tend to support this view as there is no very significant difference, as far as we know, between the pathological syndrome of staphylococcal infections in sheep and in other animals whose plasmas are fully coagulable. Coagulase production by a pathogenic staphylococcus may therefore be no more than an interesting physical phenomenon which may be little concerned in the pathological process.

Strains preserved by desiccation have appeared to lose some of their coagulative powers as shown on test after re-culture; this was made evident chiefly by comparison of the strength of the reaction produced by freshly isolated human strains and that produced by desiccated strains of human origin after re-culture. Our main object, in any case, appeared to have been fully achieved in so far as we have demonstrated that the coagulase reaction is confined to the pathogenic staphylococci of whatever animal source of origin. The slide test is a ready means of identifying these organisms. Furthermore, human, ox, mouse, rabbit and cat plasmas can be used indifferently in

the test. Pig and dog plasmas may miss a few strains, while those of the horse, sheep and guinea-pig are quite unreliable.

The strains isolated from the goslings were peculiar in that neither test for haemolysis on blood agar nor the demonstration of a filtrable haemolysin could be accepted as a criterion of pathogenicity.

As we have indicated, little if any advantage is gained by classifying staphylococci according to their capacity for producing pigment but the genus might well be divided according to Fairbrother's classification into *Staphylococcus pyogenes* and *Staphylococcus saprophyticus* using coagulase production as a basis for the division.

SUMMARY

Coagulase slide tests have been carried out on food, human and animal strains of staphylococci with human and animal plasmas.

Coagulase production is confined to pathogenic strains of staphylococci and this test is specific for the identification of these strains.

Plasmas can be used from human, ox, mouse, rabbit and cat species for slide test. Very occasional strains may not be detected by dog and pig plasmas, while horse, sheep and guinea-pig plasmas have been found most unreliable.

On the basis of previous work and together with the evidence presented it is suggested that organisms of the genus Staphylococcus may be divided into coagulase-positive strains (*Staphylococcus pyogenes*) and coagulase-negative strains (*Staphylococcus saprophyticus*), and that division on the basis of pigment production may well be discontinued.

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REFERENCES

Berger, F. M. (1943). *J. Path. Bact.*, **55**, 435.
Birch-Hirschfield, L. (1944). *Klin. Wschr.*, **13**, 331.
Cadness-Graves, B., Williams, R., Harper, G. J., and Miles, A. A. (1943). *Lancet*, **244**, 736.
Cowan, S. T. (1938). *J. Path. Bact.*, **46**, 31.
Cruickshank, R. (1937). *Ibid.*, **45**, 295.
Fairbrother. (1940). *Ibid.*, **50**, 83.
Fisk, A. (1940). *Brit. J. exp. Path.*, **21**, 311.
Gillespie, G. H. (1943). *Publ. Hlth. Serv. Bull.*, **2**.
Much, H. (1908). *Biochem. Zeitschr.*, **14**, 143.
Smith, W., and Hale, J. H. (1944). *Brit. J. exp. Path.*, **35**, 101.

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COMPARISON OF THE IMMUNISING VALUE IN GUINEA-PIGS OF LIVING AVIRULENT *BRUCELLA ABORTUS* VACCINES, STRAINS 45(20) AND S.19

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INTRODUCTION

Attempts to immunise guinea-pigs against infection with *Brucella abortus* by means of injections of dead or living suspensions of the organism have been described by several workers, Huddleson (1924), Schroeder and Cotton (1925), Torrey and Hallman (1930), Gwatkin (1931), McEwen and Roberts (1936), McEwen and Priestley (1938). Most recent are the studies of McEwen and Priestley (1940) who compared the immunity produced in guinea pigs by vaccination with living attenuated strains of *Br. abortus* with that obtained with dead vaccines. These workers concluded that vaccination with *Br. abortus* strain 45 followed by strain 45(20) gave the most satisfactory immunity: that all the living (attenuated) vaccines used gave a better immunity than the dead ones, though dead vaccines did confer some degree of immunity: that, in the case of living vaccines, resistance may drop slightly when the time elapsing between vaccination and exposure to infection is relatively great.

The experiments to be described in this paper covered a similar field to those of McEwen and Priestley. They were undertaken to compare the efficacy of vaccines prepared from living suspensions of *Br. abortus* strains 19 and 45(20), using the guinea-pig as experimental animal. The behaviour of both the above strains in the guinea-pig has been described by other workers [Cotton, Buck and Smith, (1933); McEwen and Priestley, (1938)]. They have both been designated as strains of reduced virulence, lacking the capacity to multiply or produce lesions in the guinea-pig. Strain 19 is a smooth strain; 45(20) is rough. Both are capable of growing aerobically.

In these experiments information was sought regarding the fate of (1) these avirulent strains within the guinea-pig: (2) a strain of high virulence, Strain 544, (McEwen 1940) within the guinea-pig: (3) the fully virulent strain within guinea-pigs which had previously been vaccinated with one or other of the avirulent strains.

Different methods of vaccination were employed, the variations being in the size of dose, the frequency of application and the time between vaccination and exposure to virulent infection.

Methods

Sources of strains of Br. abortus employed. Strain 45 (20) was grown from a dried culture supplied by Dr. McEwen; strain 19 was isolated from the S19 vaccine issued by the Ministry of Agriculture. Strain 544 was obtained from Dr. McEwen and passaged three times through guinea-pigs which resulted in a slight enhancement of its virulence for this animal.

Dried cultures of all three strains were prepared in large numbers and all suspensions used in this work were made from subcultures of this dried material.

Preparation of suspensions of Br. abortus. Dried cultures when required for use were suspended in broth and transferred to bacto-tryptose agar slopes. After three days' incubation in an atmosphere of 10 per cent. CO_2 at 37° C. they were subcultured and grown for a further forty-eight hours under the same conditions. The resultant growth was washed from the slopes with Ringer's solution and the opacity of the suspension adjusted to match that of number 2 tube, Brown's scale. Suspensions standardised in this way were found to have an average viable count of three thousand million organisms, as determined by dilution and colony counting. These standardised suspensions were then adjusted by dilution to contain the requisite number of viable organisms per c.c. Viable counts were always made on all suspensions employed in this work.

Inoculation of guinea-pigs. Male guinea-pigs of about 350 g. weight were used except where otherwise stated. Vaccines were injected intramuscularly into the thigh of the right leg. Suspensions of the virulent test strain were inoculated intramuscularly into the left leg.

Assessment of degree of infection in guinea-pigs

(*a*) *Viable count of spleen.* The estimated number of viable organisms in the spleen was taken as the main index of infection. For the purpose of making this count the spleen was removed, placed in a weighed Griffith's tube, its weight determined and twice its weight of sterile Ringer's solution added. After thorough grinding the resulting suspension of spleen tissue was diluted 1/10, 1/100 and 1/1,000. 0·1 c.c. amounts of the original suspension and of the dilutions were spread on blood agar plates and incubated for four to six days in an atmosphere of 10 per cent. CO_2. The number of colonies on all plates showing less than 400 were counted and the approximate number of organisms in the whole spleen was calculated from such counts. These estimations of the viable count were affected by errors due to imperfect grinding of the spleen tissue and random sampling. Special experiments to determine the probable magnitude of the combined grinding and sampling errors showed that the estimated spleen counts are likely to err by ± 20 per cent.

(*b*) *Percentage spleen weight.* Infection of guinea-pigs with *Br. abortus* produces splenic enlargement. The spleen weight was therefore obtained for every animal used in this work and, expressed as a percentage of the body weight, was taken as a secondary index of infection.

(*c*) *Agglutination titre of serum.* Blood was obtained from the heart or prescapular vein of each animal killed and dilutions of serum ranging from 1/5 to 1/10,000 were prepared. The antigen employed was that issued by the Ministry of Agriculture. Tubes containing the antigen and serum dilution were incubated at 37° C. for 18 hours and read to a standard endpoint which was the last tube in which detectable agglutination could be observed.

RESULTS

Behaviour of the vaccine strains in guinea-pigs

Suspensions of *Br. abortus* strains 19 and 45(20) were standardised to contain three million viable organisms per c.c. Groups of guinea-pigs were inoculated intramuscularly with one c.c. of this suspension. They were killed at various intervals after inoculation and their spleens examined for *Br. abortus*. Tables I and II show the amount

TABLE I

COURSE OF INFECTION IN GUINEA-PIGS INOCULATED INTRAMUSCULARLY WITH THREE MILLION VIABLE ORGANISMS OF *Brucella abortus* STRAIN 45(20)

	Time from Inoculation									
	3 *Days*	5 *Days*	7 *Days*	11 *Days*	16 *Days*	3 *Weeks*	6 *Weeks*	9 *Weeks*	12 *Weeks*	15 *Weeks*
Spleen counts	0	18	130	300	2,100	3,300	4,400	250	1,400	0
	0	0	0	600	3,500	15,000	1,900	1,800	0	0
	0	11	130	1,700	6,400	750	920	1,200	80	0
	0	14	25	200	500	18,000	1,000	0	10	0
	0	0	12	70	10,000	500	6,500	750	0	0
							33,000	0	20	0
							450			0
							500			0
							10,000			0
							1,000			0
Mean spleen counts ...	0	9	60	600	4,500	7,500	6,000	700	250	0
Mean % spleen weights	0·09%	0·10%	0·09%	0·12%	0·11%	0·09%	0·11%	0·10%	0·12%	0·10%
Mean aggl. titres ...	N	N	N	N	N	N	N	N	N	N

TABLE II

COURSE OF INFECTION PRODUCED IN GUINEA-PIGS BY THE INTRAMUSCULAR INOCULATION OF THREE MILLION VIABLE ORGANISMS OF *Brucella abortus* STRAIN 19

	Time from Inoculation.									
	3 *Days.*	5 *Days.*	7 *Days.*	11 *Days.*	16 *Days.*	3 *Weeks.*	6 *Weeks.*	9 *Weeks.*	12 *Weeks.*	15 *Weeks.*
Spleen counts	340	0	89,000	0	7,300	170	0	0	0	0
	660	10,000	370	670	4,400	270	0	0	0	0
	370	130	440	0	0	0	0		0	0
	60	790	900	100	200	1,100	0		0	0
	160	560	45,000	69,000	0	0	0		0	0
							0		0	
							0		0	
							0		0	
Mean spleen counts ...	320	2,300	27,000	14,000	2,300	310	0	0	0	0
Mean % spleen weights	0·11%	0·10%	0·13%	0·12%	0·13%	0·13%	0·10%	0·13%	0·10%	0·11%
Mean aggl. titres ...	N	N	N	1/20	1/80	1/40	N	N	N	N

TABLE III

THE COURSE OF INFECTION IN GUINEA-PIGS PRODUCED BY THE INTRAMUSCULAR INOCULATION OF THREE HUNDRED AND THREE MILLION VIABLE ORGANISMS OF *Brucella abortus* Strain 544

	Time from Inoculation.										
	3 *Days.*	5 *Days.*	7 *Days.*	11 *Days.*	16 *Days.*	3 *Weeks.*	6 *Weeks.*	9 *Weeks.*	12 *Weeks.*	15 *Weeks.*	30 *Weeks.*
	Dose Three Million Viable Organisms.										
Mean spleen counts ...	17,000 ±3,000	36,000 ±10,000	50,000 ±9,000	380,000 ±50,000	2,000,000 ±100,000	660,000 ±180,000	800,000 ±150,000	820,000 ±100,000	140,000 ±90,000	7,000 ±4,000	0
Mean % spleen weights	0·10% ±0·01	0·10% ±0·01	0·11% ±0·01	0·13% ±0·02	0·17% ±0·01	0·20% ±0·04	0·43% ±0·05	0·82% ±0·08	0·75% ±0·05	0·43% ±0·05	0·11% ±0·01
Mean aggl. titre ...	N	N	1/10	1/160	1/640	1/640	1/1,280	1/10,000	1/10,000	1/2,560	1/1,280
Number of guinea-pigs	5	5	5	5	20	5	9	20	17	4	5
	Dose Three Hundred Viable Organisms.										
Mean spleen counts ...		100			12,000 ±5,000			100,000 ±11,000			
Mean % spleen weights		0·11% ±0·02			0·13% ±0·01			0·55% ±0·02			
Mean aggl. titre ...		N			1/40			1/5,120			
Number of guinea-pigs		5			5			8			

and duration of infection produced by these two vaccine strains. The data indicate that strain 45(20), though slower to make an appearance in the spleen, persisted there for a longer period than did strain 19. Neither strain produced any significant increase in the percentage weight of the spleen. Agglutinins to low titre were demonstrable in the sera of animals inoculated with strain 19. Strain 45(20) did not produce agglutinins demonstrable by means of the smooth antigen used in these experiments.

Both strains can therefore be described as strains of low virulence for the guinea-pig incapable of multiplying extensively in its tissues or of producing observable lesions in its organs.

Behaviour of strain 544 *in unvaccinated guinea-pigs*

Two groups of guinea-pigs were inoculated intramuscularly with one c.c. amounts of suspensions of *Br. abortus* strain 544. The first group received a dose containing about three million viable organisms, the other received about three hundred viable organisms. These guinea-pigs were killed in batches at various intervals after inoculation. Table III shows the amount of infection produced by this strain, the mean spleen counts being accompanied, in each case, by their standard deviations. It will be seen that strain 544 multiplied actively within the spleens of these guinea-pigs even when inoculated in small numbers. It could still be recovered from the spleens of infected animals at fifteen but not at thirty weeks after their exposure to infection. The infection caused a progressive enlargement of the spleen which attained a maximum about nine weeks after exposure to infection. It stimulated the production of agglutinins to high titre which persisted after the bacteria themselves had been eliminated.

Strain 544 can therefore be described as a strain of high virulence for the guinea pig capable of invading and multiplying in the animal's tissues, of producing considerable splenic enlargement and agglutinins to a high titre. In all these respects it differs from strains 19 and 45(20).

Behaviour of strain 544 *in vaccinated guinea-pigs*

Influence of size and frequency of vaccine dose.—Suspensions of living organisms were prepared from *Br. abortus* strains 19 and 45(20) and the following vaccination treatments given to groups of 15 guinea-pigs.

Treatment A.—One dose containing three thousand million viable organisms.

Treatment B.—One dose containing three hundred thousand viable organisms.

Treatment C.—Three doses at fortnightly intervals each containing three thousand viable organisms.

Treatment D.—Three doses at fortnightly intervals containing three thousand, three million and three thousand million viable organisms respectively.

In the case of those guinea-pigs which received vaccination with strain 19 the development of agglutinins subsequent to vaccination

was followed by taking blood samples at weekly intervals. Mean values of these agglutination titres are given in Table IV which suggests that, with each successive vaccination, the response of the animal has decreased.

TABLE IV

MEAN AGGLUTINATION TITRES (RECIPROCALS) OF GUINEA-PIG'S SERA FOLLOWING VACCINATON WITH VACCINES PREPARED FROM LIVING SUSPENSIONS OF *Brucella abortus* STRAIN 19

Vaccination Treatment.	*Weeks from First Dose of Vaccine.*									
	1	2	3	4	5	6	7	8	9	10
One dose 3×10^9 v. orgs. ...	40	80	320	80	40	40				
One dose 3×10^5 v. orgs. ...	N	80	80	80	40	10				
Three doses 3×10^3 v. orgs. each	N	40	40	80	20	10	N	N	N	N
Three doses 1. 3×10^3 v. orgs. ... 2. 3×10^6 v. orgs. ... 3. 3×10^9 v. orgs. ...	N	40	80	40	40	80	40	40	20	10

Six weeks after the last injection of vaccine the animals were exposed to infection with strain 544. Three sizes of test dose were used containing 3×10^2, 3×10^5 and 3×10^8 viable organisms respectively. Five animals from each vaccinated group were inoculated with each test dose. Groups of unvaccinated guinea-pigs were inoculated with the three test doses at the same time.

For nine weeks following exposure to these test doses blood samples were drawn at weekly intervals from all the one hundred and fifty animals used in this experiment. Mean agglutination titres are shown in Table V. Since it appears that agglutination titre and spleen count are to some extent correlated (see Table XI) these figures can be taken as approximately reflecting the progress of infection in vaccinated and unvaccinated animals. Judged by this criterion it is evident that vaccination has reduced the amount of infection in almost all the groups. But agglutination titres cannot be relied on to indicate the exact degree of infection while, in the case of guinea-pigs vaccinated with strain 19, the interpretation of results is further complicated by the persistence of agglutinins due to the vaccine itself. In general the agglutination titres of animals vaccinated with strain 19 were higher than were those of animals in the same group vaccinated with 45(20). This, however, does not indicate a higher degree of infection in the strain 19 vaccinated animals, as will be shown later.

Nine weeks after injection of the fully virulent strain all the guinea-pigs were killed and the degree of infection estimated for each animal by determining its spleen count. For the purpose of statistical treatment these spleen counts have been grouped into six classes ranging from 0 to over 10^6 and the distribution of their frequencies is shown in Table VI. By adding together frequencies

TABLE V

MEAN SERUM AGGLUTINATION TITRES (RECIPROCALS) OF GROUPS OF VACCINATED AND UNVACCINATED GUINEA-PIGS FOLLOWING EXPOSURE TO INFECTION WITH *Brucella abortus* STRAIN 544

Test Dose.	*Vaccine.*	*Treatment.*	*Weeks from Test Dose.*								
			1	2	3	4	5	6	7	8	9
3×10^8 v. orgs.	S19	A	80	640	640	2,560	2,560	1,280	1,280	2,560	5,120
		B	40	320	320	640	640	640	1,280	2,560	2,560
		C	20	320	640	1,280	1,280	1,280	2,560	1,280	1,280
		D	40	320	640	640	640	1,280	1,280	1,280	1,280
	45(20)	A	20	80	80	640	640	640	1,280	1,280	1,280
		B	40	160	640	640	1,280	2,560	5,120	—	10,000
		C	40	40	40	160	640	—	640	640	640
		D	80	80	160	320	320	640	640	—	640
3×10^5 v. orgs.	S19	A	20	40	160	320	320	320	320	640	1,280
		B	N	40	320	640	640	320	320	320	640
		C	N	160	320	320	320	640	640	640	640
		D	20	80	640	640	640	640	640	640	320
	45(20)	A	N	10	20	80	80	20	80	80	80
		B	N	40	160	320	640	1,280	1,280	—	5,000
		C	N	20	20	40	40	80	80	80	80
		D	N	40	40	160	160	160	160	160	160
3×10^2 v. orgs.	S19	A	N	20	20	10	10	10	10	10	10
		B	N	N	N	N	20	20	20	20	20
		C	N	N	N	N	10	10	10	10	10
		D	40	20	20	20	40	80	80	80	80
	45(20)	A	N	N	N	40	40	40	40	40	40
		B	N	N	10	80	80	160	160	—	160
		C	N	N	N	N	40	80	80	80	80
		D	N	N	N	N	N	N	N	N	N
3×10^8 unvaccinated			80	320	640	1,280	2,560	5,000	5,000	10,000	20,000
3×10^5 „			N	160	640	640	1,280	2,560	5,000	10,000	20,000
3×10^2 „			N	N	40	80	160	640	1,280	5,000	10,000

* Each value a mean of five to eight determinations.

of spleen counts above and below a value of 10^3 the following figures were obtained :—

Treatment.	S19 Vaccine.		45(20) Vaccine.		Unvaccinated.	
	Under 10^3	Over 10^3	Under 10^3	Over 10^3	Under 10^3	Over 10^3
A	9	5	6	8	—	—
B	8	5	5	9	—	—
C	10	5	6	7	—	—
D	12	2	6	9	—	—
Totals	39	17	23	33	1	34

In the case of each of the vaccinated groups the amount of infection has been significantly reduced as compared with that in the unvaccinated groups. On comparing the various treatments amongst themselves the following values for χ^2 and P were obtained :—

S19 Vaccine.			45(20) Vaccine.			S19 versus 45(20) Vaccine.		
Treatment.	χ^2	P	Treatment.	χ^2	P	Treatment.	χ^2	P
A v. B	0·022	0·9	A v. B	0·150	0·7	A v. A	1·294	0·3
A v. C	0·018	0·9	A v. C	0·029	0·95	B v. B	1·80	0·2
B v. C	0·115	0·7	B v. C	0·324	0·7	C v. C	1·19	0·3
A v. D	1·71	0·2	A v. D	0·024	0·95	D v. D	6·69	0·01
C v. D	1·47	0·3	C v. D	0·107	0·8	Total S19 v. Total 45(20)	9·28	0·01
B v. D	2·04	0·2	B v. D	0·047	0·9			

In the group vaccinated with strain 19, treatment D showed an apparent superiority over the others but it is not sufficiently large to be considered significant. In the group vaccinated with 45(20) all four treatments were of approximately equal value. Comparing the effect of the strain 19 vaccines with that of the 45(20) vaccines it is evident that strain 19 gave rather better protection in every instance, the difference being highly significant in the case of treatment D. When the total spleen count distributions are compared it becomes clear that the protection afforded by strain 19 vaccines was very significantly better than the protection afforded by the 45(20) vaccines. It is of interest to note that, in the case of both vaccines, the injection of three small doses of vaccine at fortnightly intervals (treatment C) gave as good an immunity as the injection of a single dose containing over three hundred thousand times as many organisms (treatment A).

Influence of time elapsing between vaccination and exposure to test strain.—Two groups each of 72 guinea-pigs were vaccinated, the one with a suspension of strain 19 the other with a suspension of strain 45(20). The vaccine dose contained 3×10^6 viable organisms suspended in one c.c. of Ringer's solution. These animals were subdivided into three equal units and exposed to infection with

TABLE VI

FREQUENCY DISTRIBUTION OF SPLEEN COUNTS IN VACCINATED AND UNVACCINATED GUINEA-PIGS KILLED NINE WEEKS AFTER EXPOSURE TO INFECTION WITH *Brucella abortus* STRAIN 544

Treatment.	*Spleen Counts.*							
	0	1 to 100	100 to 1,000	1,000 to 10,000	10,000 to 100,000	100,000 to 1,000,000	Over 1,000,000	*Totals.*
S19 Vaccine								
Treatment A	4	0	5	2	2	1		14
„ B	3	3	2	4	1			13
„ C	4	2	4	4	1			15
„ D	5	2	5	1	1			14
45(20) Vaccine								
Treatment A	5	0	1	3	3	2		14
„ B	2	0	3	1	3	4	1	14
„ C	5	1	0	2	3	2		13
„ D	3	1	2	3	3	3		15
Unvaccinated controls			1	2	13	12	10	38

TABLE VII

FREQUENCY DISTRIBUTION OF SPLEEN COUNTS OF GUINEA-PIGS EXPOSED TO INFECTION WITH *Brucella abortus* STRAIN 544 AT DIFFERENT INTERVALS AFTER VACCINATION

Treatment.	*Spleen Counts.*							
	0	1 *to* 100	100 *to* 1,000	1,000 *to* 10,000	10,000 *to* 100,000	100,000 *to* 1,000,000	*Over* 1,000,000	*Totals.*
Tested 3 weeks after vaccination								
S19 vaccine	3	2	2	2	3			12
45(20) vaccine	1	0	1	3	2	5		12
Unvaccinated					4	5	3	12
Tested 7 weeks after vaccination								
S19 vaccine	4	4	2	0	0	2		12
45(20) vaccine	3	1	1	3	4			12
Unvaccinated				2	3	4	3	12
Tested 14 weeks after vaccination								
S19 vaccine	3	1	2	0	3	3		12
45(20) vaccine	5	0	1	4	1	1		12
Unvaccinated				2	3	4	3	12

Br. abortus strain 544 at three weeks, seven weeks and 14 weeks after vaccination. Half the animals in each unit were left unexposed to the virulent strain to provide information regarding the persistence of the vaccine strain. Two sizes of test dose were used, one containing 3×10^6, the other 3×10^3 viable organisms. The animals were killed in equal numbers at two weeks and 12 weeks after exposure to strain 544 and their spleen counts, per cent. spleen weights and agglutination titres determined. Vaccinated animals which had not been exposed to strain 544 were killed at the same time and corrections made in the spleen counts to allow for organisms of the vaccine strain still persisting in the spleen.*

Table VII shows the frequency distribution of spleen counts in the three groups of guinea-pigs exposed to infection with strain 544 at different intervals after vaccination. Data from animals receiving large and small test doses and killed at two and 12 weeks after exposure to infection have been combined. Summing frequencies above and below a spleen count value of 10^3 gives the following figures :—

Interval between Vaccination and Test Dose.	*S19 Vaccine.*		*45(20) Vaccine.*		*Unvaccinated.*	
	Under 10^3	*Over* 10^3	*Under* 10^3	*Over* 10^3	*Under* 10^3	*Over* 10^3
3 weeks ...	7	5	2	10	0	12
7 „ ...	10	2	5	7	0	12
14 „ ...	6	6	6	6	0	12

To compare the distribution of spleen counts in vaccinated and unvaccinated animals by the χ^2 test it was necessary to sum the frequencies above and below a spleen count of 10^4. When this was done even the poorest vaccine treatment (45(20), interval three weeks) gave a value for P of less than ·05 when compared with the controls. A comparison of the vaccine treatments amongst themselves gave the following values of χ^2 and P.

S19 Vaccine Treatments.			*45(20) Vaccine Treatments*			*S19 v. 45(20).*		
Treatment.	χ^2	*P*	*Treatment.*	χ^2	*P*	*Treatment.*	χ^2	*P*
3 v. 7 weeks	1·82	0·2	3 v. 7 weeks	1·81	0·2	3 v. 3 weeks	4·45	0·05
3 v. 14 „	0·168	0·7	3 v. 14 „	3·0	0·1	7 v. 7 „	4·45	0·05
7 v. 14 „	3·00	0·1	7 v. 14 „	0·168	0·7	14 v. 14 „	0	—

* Strain 45(20) was recovered from the spleens of those guinea-pigs killed five and nine weeks after vaccination. It was found possible to distinguish vaccine from virulent strain by incubating plates inoculated with spleen material in air for two days before placing them in an atmosphere of CO_2. At the time of counting four days later 45(20) colonies could be distinguished from 544 colonies by their greater size and opacity. This treatment did not have any effect on the 544 count. Strain 19 was not recovered from any of the vaccinated guinea-pigs at the time of killing.

It is evident from this table that strain 19 generated an immunity in guinea-pigs more rapidly than did 45(20). Resistance of guinea-pigs vaccinated with strain 19 was apparently as high at three weeks after vaccination as it was at 14 weeks after vaccination. At both three and seven weeks it was significantly higher than that of animals vaccinated with 45(20).

Effect of vaccination of guinea-pigs in infancy with a vaccine prepared from Br. abortus strain 19.—24 male and 24 female guinea-pigs aged three to four weeks, average weight 200 g. were inoculated intramuscularly with 0·5 c.c. of a suspension of *Brucella abortus* strain 19 containing 3×10^9 viable organisms per c.c. Samples of blood were taken by cardiac puncture at weekly intervals after vaccination and the agglutination titre of the serum determined. Table VIII shows the mean agglutination titres of groups of male and female animals during the first 11 weeks after vaccination.

TABLE VIII

MEAN SERUM AGGLUTINATION TITRES (RECIPOCALS) OF GROUPS OF MALE AND FEMALE GUINEA-PIGS FOLLOWING THEIR VACCINATION AT THE AGE OF THREE TO FOUR WEEKS WITH *Brucella abortus* STRAIN 19

	Weeks from Vaccination.										
	1	2	3	4	5	6	7	8	9	10	11
Male guinea-pigs ...	40	160	80	20	20	20	20	20	20	10	20
Female „ ...	40	160	160	80	40	40	40	40	20	20	20

Each value is a mean of ten determinations.

It will be seen that the production of agglutinins began within seven days from the injection of the vaccine and that agglutinins could be demonstrated in the serum during the whole 11 week period.

Three months after vaccination one vaccinated male was put in with every three vaccinated females. A group of sixteen unvaccinated females was mated at the same time to provide controls. All these female guinea-pigs were exposed to infection with strain 544 as soon as pregnancy could be demonstrated. The group of males was exposed to infection at about the same time, sixteen unvaccinated males comprising the controls. Two doses of the test strain were employed containing 3×10^5 and 3×10^2 viable organisms respectively. A group of 12 vaccinated animals and eight unvaccinated animals of each sex was inoculated with the large test dose and a similar group with the small dose.

An attempt was made to observe the number of abortions amongst the pregnant female animals vaccinated and unvaccinated but this proved impossible to determine with accuracy. The number of live young was recorded instead and is shown in Table IX. The difference in numbers of live young shown in the table suggests

TABLE IX

NUMBER OF LIVE OFFSPRING PRODUCED BY GROUPS OF VACCINATED AND UNVACCINATED GUINEA-PIGS EXPOSED DURING PREGNANCY TO INFECTION WITH *Brucella abortus* STRAIN 544

	Test Dose.	*Number of Pregnant Females.*	*Number of Live Offspring.*
Vaccinated ...	3×10^5 v. orgs.	12	4
	3×10^3 v. orgs.	12	11
	Total ...	24	15
Unvaccinated	3×10^5 v. orgs.	8	1
	3×10^3 v. orgs.	7	0
	Total ...	15	1

that vaccine treatment significantly reduced the rate of abortion.

Nine weeks after exposure to infection with strain 544 all the animals were killed and their spleen counts, per cent. spleen weights and agglutination titres determined. In Table X the frequency distribution of spleen counts in vaccinated and unvaccinated animals

TABLE X

FREQUENCY DISTRIBUTION OF SPLEEN COUNTS IN MALE AND FEMALE GUINEA-PIGS VACCINATED AT THREE TO FOUR WEEKS OF AGE WITH *Brucella abortus* STRAIN 19 AND KILLED NINE WEEKS AFTER EXPOSURE TO INFECTION WITH STRAIN 544

	Spleen Counts.							
Treatment.	0	1 *to* 100	100 *to* 1,000	1,000 *to* 10,000	10,000 *to* 100,000	100,000 *to* 1,000,000	*Over* 1,000,000	*Totals.*
Vaccinated								
Males ...	12	2	1	4	3	2		24
Females ...	10	3	5	2	3	1		24
Unvaccinated								
Males ...				1	3	10	4	18
Females ...			1	1	4	4	5	15

is shown. Summing spleen count frequencies above and below 10^3 gives the following figures :—

	Vaccinated.		*Unvaccinated.*	
	Under 10^3	*Over* 10^3	*Under* 10^3	*Over* 10^3
Males	15	9	0	18
Females	18	6	1	14
Totals... ...	33	15	1	32

TABLE XI

CORRELATION OF SPLEEN COUNTS, % SPLEEN WEIGHTS AND AGGLUTINATION TITRES (RECIPROCALS) IN GUINEA-PIGS INFECTED WITH *Brucella abortus* STRAIN 544, KILLED NINE WEEKS AFTER EXPOSURE TO INFECTION

		Spleen Counts.						
		0	1 to 100	100 to 1,000	1,000 to 10,000	10,000 to 100,000	100,000 to 1,000,000	Over 1,000,000
Unvaccinated ...	Mean % spleen weights				0·26% ±0·03	0·48% ±0·06	1·10% ±0·13	2·41% ±0·22
	Mean aggl. titre ...				1,280 to 10,000	2,560 to 20,000	2,560 to 20,000	10,000 to 40,000
	Number of guinea-pigs				4	21	27	18
Vaccinated S19...	Mean % spleen weights	0·11% ±0·01	0·11% ±0·01	0·15% ±0·01	0·17% ±0·01	0·32% ±0·04	0·74% ±0·20	
	Mean aggl. titre ...	N to 20	160 to 640	320 to 2,560	320 to 5,000	640 to 10,000	5,000 to 10,000	
	Number of guinea-pigs	38	11	21	16	11	4	
Vaccinated 45(20)	Mean % spleen weights	0·13% ±0·01		0·14% ±0·01	0·18% ±0·01	0·26% ±0·02	0·84% ±0·12	
	Mean aggl. titre ...	N		80 to 640	160 to 640	320 to 1,280	2,560 to 10,000	
	Number of guinea-pigs	13		9	9	12	8	

It is clear from these figures that the vaccine treatment applied to these guinea-pigs at an age of three to four weeks conferred on them a resistance to infection with a virulent strain of *Br. abortus*, and that this enhanced resistance was still present at the time the animals reached sexual maturity. The figures also show that the animals' sex made no significant difference either to its response to vaccination or to its susceptibility to infection with the virulent strain.

Correlation of Spleen count, per cent. Spleen Weight and Agglutination Titre in guinea-pigs infected with a virulent strain of Br. abortus.

In all the above experiments the estimated viable count of the spleen has been taken as the index of infection. For every guinea-pig killed, however, a value was obtained for the spleen weight (expressed as a percentage of the body weight) and for the agglutination titre of an animal's serum. From these data the degree of correlation between spleen count, per cent. spleen weight and agglutination titre has been estimated using values obtained from over 220 vaccinated and unvaccinated animals. All these animals were killed nine weeks after exposure to infection with strain 544.

In Table XI the spleen counts have been grouped and mean values calculated for the corresponding per cent. spleen weights and agglutination titres. Each mean per cent. spleen weight is accompanied by its standard error. The agglutination titres cover a range corresponding to about twice the standard error of the mean.

It is clear from this table that a positive correlation existed between spleen counts and per cent. spleen weights both in the vaccinated and unvaccinated animals. For a group of 45 normal guinea-pigs the mean per cent. spleen weight was found to be $0{\cdot}10 \pm 0{\cdot}01$ per cent. From this it will be seen that the per cent. spleen weights in the vaccinated groups corresponding to spleen counts between 100 and 1,000 are significantly increased. It is also evident from the table that vaccination has significantly decreased the amount of splenic enlargement corresponding to any spleen count. Agglutination titres, though also positively correlated with the spleen counts, were subject to very great variation and did not constitute such a reliable index of infection as the per cent. spleen weights.

SUMMARY AND CONCLUSIONS

(1) The behaviour in the guinea-pig of *Brucella abortus* strains 19 and 45(20) was studied. Both strains were found incapable of multiplying to any extent in the spleen or of producing enlargement or lesions in this organ. After inoculation of 3×10^6 viable organisms it was found that strain 45(20) persisted in the spleen for up to 12 weeks ; strain 19 was eliminated in less than six.

(2) *Br. abortus* strain 544 was found to be of high virulence for the guinea-pig capable of invading and multiplying in the spleen in which it produced lesions and considerable enlargement. It persisted in the spleen for longer than 15 weeks and caused the

appearance of agglutinins to high titre in the blood serum which persisted after the organisms themselves had been eliminated.

(3) Vaccines prepared from suspensions of living organisms of *Br. abortus* strains 19 and 45(20) were employed for the immunisation of guinea-pigs against infection with the virulent strain 544. In general vaccines prepared from strain 19 conferred a better immunity than did vaccines prepared from strain 45(20). Three small doses of vaccine given at fortnightly intervals conferred as good an immunity as did a single dose of vaccine containing 300 thousand times as many viable organisms.

Vaccines prepared from strain 19 were found to produce immunity in guinea-pigs more rapidly than did vaccines prepared from strain 45(20).

A single dose of a vaccine prepared from strain 19 administered to guinea-pigs of between three and four weeks old conferred an immunity which persisted until the animals reached maturity.

Neither of the two vaccines proved capable of rendering a group of guinea-pigs completely resistant to infection with strain 544 even when the animals were exposed to infection with as few as 300 viable organisms of the virulent strain. In such cases 20 to 30 per cent. of the animals were always found to be infected, though the amount of infection was very small as compared with that of unvaccinated controls.

(4) A positive correlation was demonstrated between spleen count, spleen weight (expressed as a percentage of the body weight) and the agglutination titre of vaccinated and unvaccinated guinea-pigs killed nine weeks after exposure to infection with strain 544. The per cent. spleen weight corresponding to a given spleen count was consistently lower in vaccinated than in unvaccinated animals.

Agglutination titres proved a less reliable index of infection than did the per cent. spleen weights.

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REFERENCES

Cotton, W. E., Buck, J. M., and Smith, H. E. (1933). *J. agric. Res.*, **46**, 291.

Gwatkin, R. (1931). *J. inf. Dis.*, **48**, 381.

Huddleson, I. F. (1924). *Mich. agric. Coll. Exp. Sta., Tech. Bull.* 65.

McEwen, A. D., and Roberts, R. S. (1936). *J. comp. Path.*, **49**, 97.

McEwen, A. D., and Priestley, F. W. (1938). *Vet. Rec.*, **50**, 1097; (1940). *Ibid.*, **53**, 743.

McEwen, A. D. (1940). *Ibid.*, **52**, 97.

Schroeder, E. C., and Cotton, W. E. (1925). *J. Amer. vet. med. Ass.*, **66**, 551.

Torrey, J. P., and Hallman, E. T. (1930). *Ibid.*, **76**, 7.

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THE PROPAGATION OF *BRUCELLA ABORTUS* IN THE MOUSE

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INTRODUCTION

That mice can be infected with *Brucella abortus* has been demonstrated by several workers (Holth, 1911 ; Fabyan, 1912, 1913 ; Hagan, 1922). Ber (1933) has shown that the inoculation of 800,000,000 viable organisms into mice produces a septicaemic condition in these animals and that organisms can be recovered from the blood for as long as six months after exposure to infection. He states that eight or nine organisms may be sufficient to cause infection. Feldman and Olsen (1935) were able to recover *Br. abortus* from the spleens of inoculated mice and to demonstrate agglutinins in the sera of such animals. Singer and Shaw (1935) observed that large doses (300,000,000 organisms) of the porcine type of *Br. abortus* are fatal to mice within four days. Singer-Brooks (1937) comparing the pathogenicity for mice of *Br. abortus* with that of *Br. melitensis*, also found that the injection of 900,000,000 organisms of the bovine type into mice produced death within four days. Injection of 100,000 and 1,000 organisms of the bovine type was not fatal to mice and, in most cases, organisms could not be recovered from mice subjected to such treatment. Scorgie (1938) found that relatively large doses of *Br. abortus* were required to infect mice (over 28,000 viable organisms), and that even doses of 810,000,000 viable organisms did not infect 100 per cent. of the animals inoculated.

Priestley and McEwen (1938), using strains of *Br. abortus* differing in degree of virulence for guinea-pigs, found that the avirulent strains of the organism were less toxic for mice than strains of high virulence when inoculated in massive doses by the intraperitoneal route. Kolmer, Bondi and Rule (1940) were also able to show that massive doses of *Br. abortus* (about 2,000,000,000 viable organisms) will kill mice. Stableforth (unpublished) confirmed the findings of Priestley and McEwen that the toxicity of *Br. abortus* for mice gives a measure of the virulence of the strain but both workers are agreed that the mouse gives a less reliable differentiation of strains than does the guinea-pig.

The experiments to be described were designed to provide information regarding the propagation of *Br. abortus* within the tissues of the mouse. Attention was directed to the influence of the following factors :—

(1) Virulence of strain. Two strains of *Br. abortus* were tested ; strain 544, of high virulence for cattle and guinea-pigs, and strain 19, of low virulence for these animals.

(2) Route of inoculation. Four routes of inoculation were studied, comprising the intramuscular, intraperitoneal, intracerebral and intravenous.

(3) Number of organisms inoculated. Two sizes of dose were employed ; a large dose containing 300,000 viable organisms and a small dose containing 300 viable organisms.

The propagation of the organism was followed by determining the degree of infection in the livers, spleens and brains of mice at intervals of one, three and five weeks from inoculation. Histological changes produced by the parasite in the host tissue were also studied.

The design of the experiment was factorial, the 480 mice used being evenly distributed between all the treatments in the following manner :—

Two levels of dose : 240 mice at each dose level.

Two strains of organism : 120 mice at each strain-dose level.

Four routes of inoculation : 30 mice at each route-strain-dose level.

Three times of killing : 10 mice at each time-route-strain-dose level.

In the analysis of results the total distribution of degrees of infection was partitioned amongst the different variables and the chi squared test applied to determine whether any observed differences were significant. Only the main effects were analysed, *i.e.*, size of dose, strain, route of inoculation and time of killing. Interactions such as that between size of dose and strain of organism were not considered.

EXPERIMENTAL

Four hundred and eighty white mice of approximately the same weight and age were used in these experiments. The two strains of *Br. abortus* employed (strain 19 and 544) were both grown from dried cultures the virulence of which had been checked in guinea-pigs by the method described by de Ropp (1945) and shown to be characteristic for both strains.

(*a*) *Preparation of infective suspensions.* The dried cultures were suspended in broth and transferred to bacto-tryptose agar. After three days' incubation in an atmosphere of 10 per cent. CO_2 they were subcultured and incubated for a further 48 hours. The resulting growth was suspended in Ringer's solution and its opacity standardised against that of number two tube, Brown's scale. This suspension was found to contain about 3,000,000,000 viable organisms per c.c. For inoculation into mice it was diluted in such a way as to contain 300,000 or 300 viable organisms in the volume of liquid to be injected. This volume, in the case of intraperitoneal, intramuscular and intravenous inoculation was 0·1 c.c. In the case of intracerebral inoculation it was 0·05 c.c. The number of viable organisms in the inoculum was in each case checked by plate counts.

(*b*) *Assessment of degrees of infection.* At intervals of one, three and five weeks after inoculation ten mice were removed from each inoculated group and killed with chloroform. The livers, spleens and brains were removed entire and placed separately in small Griffith's tubes. The plunger of the Griffith's tube was attached by rubber tubing to the spindle of a vertical electric motor and the tissues finely ground in the presence of approximately twice their volume of Ringer's solution : 0·1 c.c. quantities of the resulting suspensions were spread on blood agar plates and incubated in an atmosphere of 10 per cent. CO_2 for from four to six days. The number of colonies of *Br. abortus* on each plate was represented by plus signs, the

following divisions being adopted: N. No *Brucella* colonies; + One to ten colonies; ++ Ten to about 500 colonies; +++ 500 colonies to almost confluent growth; ++++ Confluent growth.

(*c*) *Assessment of agglutination titre of blood serum.* At the fifth week after inoculation a sample of blood was drawn from each mouse at the time of killing. A suitable series of dilutions was prepared from the serum and mixed with an equal part of the stock *Brucella* antigen supplied by the Ministry of Agriculture. Readings were made after 18 hours' incubation at 37° C.

(*d*) *Histological investigation of tissues from infected mice.* Mice inoculated intracerebrally with 300,000 viable *Br. abortus* organisms provided the material for this study. Their spleens, livers and brains were removed at intervals of one and five weeks after inoculation, fixed in formalin and used for the preparation of sections.

RESULTS

Four main effects were studied in the present series of experiments. They were the influence on degree of infection of virulence of strains, routes of inoculation, size of infecting dose and time elapsing between inoculation and assessment of infection. For the determination of these effects, the frequency distributions of degrees of infection in the livers and spleens of the relevant groups of mice were compared. The degree of infection in the brain was studied separately.

Effect of virulence of strains.—Table I shows the frequency distribution of levels of infection in groups of mice inoculated with *Br. abortus* strain 544 and 19 respectively.

TABLE I

INFLUENCE OF STRAIN OF ORGANISM ON DEGREE OF INFECTION

Strain.	*Degrees of Infection.*				
	N	+	++	+++	++++
544	84	101	123	58	76
19	127	84	100	56	51

It will be noted that a larger proportion of observations fell in the higher categories of infection in the case of mice inoculated with strain 544 than was the case with mice inoculated with strain 19. This difference is significant at the 1 per cent. level (chi squared = 18·1 for which P lies between ·01 and ·001). It appears, therefore, that strain 544 propagated more readily in the mouse than did strain 19. But this difference between the two strains is much less obvious in the mouse than it is in the cow or guinea-pig.

Effect of route of inoculation.—Table II shows the frequency distribution of levels of infection within four groups of mice inoculated with *Br. abortus* (strains 544 and 19) by four different routes.

TABLE II

INFLUENCE OF ROUTE OF INOCULATION ON DEGREE OF INFECTION

Route of Inoculation.	*Degrees of Infection.*				
	N	+	++	+++	++++
Intramuscular ...	102	42	51	18	13
Intraperitoneal...	62	56	51	21	15
Intravenous ...	25	40	72	35	58
Intracerebral ...	22	47	49	41	41

The slightly higher degree of infection in the intraperitoneal as compared with the intramuscular group is not quite significant at the 1 per cent. level (chi squared = 10·00 for which P lies between ·05 and ·02). A comparison between the amount of infection in the intravenous and intracerebral groups reveals no significant difference (chi squared = 6·91 for which P lies between ·2 and ·1). If the frequencies of these four groups are combined, intramuscular being added to intraperitoneal and intracerebral to intravenous the difference between the two resulting distributions is highly significant (chi squared = 134, P = less than ·001). It is thus evident that *Br. abortus* propagates in the mouse very much more readily when introduced by the intravenous or intracerebral route than when introduced by the intramuscular or intraperitoneal route.

Effect of size of dose.—Table III shows the frequency distribution of levels of infection within groups of mice inoculated with different doses of *Br. abortus*.

TABLE III

INFLUENCE OF SIZE OF DOSE ON DEGREE OF INFECTION

Size of Dose.	*Degrees of Infection.*				
	N	+	++	+++	++++
300,000 v. orgs.	43	67	125	95	123
300 v. orgs.	178	118	98	14	4

The greater degree of infection produced by the larger dose is obviously highly significant. By the two less favourable routes the smaller dose of 300 organisms generally failed to infect mice, although it invariably did so by the two more favourable routes. With the larger dose some degree of infection was always produced whatever the routes of inoculation. These findings all lend support to the view that the mouse is far less susceptible to infection with *Br. abortus* than is the guinea-pig.

Rate of progress of Infection.—Table IV shows the frequency distribution of levels of infection within groups of mice killed one, three and five weeks after inoculation with *Br. abortus*.

TABLE IV

INFLUENCE OF TIME OF KILLING AFTER INOCULATION ON DEGREE OF INFECTION

Time of Killing After Inoculation.	*Degrees of Infection.*				
	N	+	++	+++	++++
One week ...	65	61	78	45	35
Three weeks ...	67	80	89	28	46
Five weeks ...	79	54	66	40	46

Comparison of the amount of infection at one week with that at three and five weeks gives values for chi squared of 7·49 and 4·60 respectively, corresponding to values for P of between ·5 and ·1. Thus it appears that, having attained a certain magnitude within the first week after inoculation, the infection neither increased nor decreased to any significant extent during the subsequent four weeks. This statement holds good for both strains at both levels of dosage. No information is provided by these data as to how soon an infection with *Br. abortus* begins to retrogress in the mouse. Ber (1933) has stated that the blood of infected mice remains infected for as long as six months after inoculation with *Br. abortus*.

Course of infection subsequent to intracerebral inoculation.—Certain special features characterised the infection produced by inoculating mice intracerebrally with *Br. abortus*. Table V shows the frequency distribution of levels of infection in mice killed one, three and five weeks after intracerebral inoculation with *Br. abortus*. The amounts of infection in brains, livers and spleens are shown separately.

TABLE V

DISTRIBUTION OF INFECTION AMONGST DIFFERENT ORGANS IN INFECTED MICE

Organ.	*Weeks from Inoculation.*														
	One Week.					*Three Weeks.*					*Five Weeks.*				
	N	+	++	+++	++++	N	+	++	+++	++++	N	+	++	+++	++++
Brain	0	0	9	21	2	0	6	19	12	0	7	3	16	4	0
Spleen	0	8	6	8	12	1	6	5	5	17	1	2	8	12	9
Liver	6	8	13	7	0	10	13	8	5	0	6	10	9	5	1

It will be observed that, one week after inoculation, a heavy infection developed in the brain. This was accompanied by an even heavier infection in the spleen and a lighter infection in the liver. It is clear, therefore, that the parasite was able to spread rapidly from the site of inoculation to these other organs. It will also be observed that, whereas the amount of infection in the liver and spleen remained more or less constant during the whole five week period, in the brain it decreased significantly both at three weeks and five weeks. Evidently the brain did not offer as suitable a medium as the spleen for the continued existence of the parasite.

Brain cultures from mice inoculated by the other three routes gave 30 positive cultures out of 175 brains examined. The number of organisms recovered, however, was invariably very small. They were probably present in the blood removed with the brain rather than in the brain tissue itself. There was no evidence to suggest that *Br. abortus* will localise and multiply in the brain of the mouse unless deliberately introduced into that organ.

Despite the extensive infection of the brain produced in mice inoculated intracerebrally with 300,000 viable organisms no deaths due to this infection occurred.

Distribution of infection between liver and spleen.—Table VI shows the frequency distribution of levels of infection as between livers and spleens of mice infected with *Br. abortus*.

TABLE VI

COMPARATIVE DISTRIBUTION OF INFECTION BETWEEN LIVERS AND SPLEENS

Organ.	*Degrees of Infection*				
	N	+	++	+++	++++
Spleen	67	66	117	71	108
Liver	139	116	108	53	17

The greater degree of infection of the spleen shown by these figures is highly significant and was invariably observed in all the mice used in this experiment.

Agglutination titres of sera of infected mice.—Table VII shows the distribution of agglutination titres within groups of infected mice killed five weeks after inoculation with 300,000 viable organisms.

TABLE VII

INFLUENCE OF STRAIN AND ROUTE OF INOCULATION ON DISTRIBUTION OF AGGLUTINATION TITRES

Route.	*Strain.*	*Agglutination Titres.*						
		N	1/20	1/40	1/80	1/160	1/320	1/640
Intramuscular ...	544	3	2	0	4	0	1	
	19	4	1	2	1	1	1	
Intraperitoneal ...	544	1	3	0	2	2	1	1
	19	6	0	1	1	2		
Intravenous ...	544	0	0	2	1	4	1	1
	19	0	1	1	1	0	4	2
Intracerebral ...	544	0	0	2	2	0	3	3
	19	0	0	0	2	5	0	3

The total distributions for the two strains do not differ significantly (chi squared=6·90 corresponding to a value for P of between ·5 and ·3). from which it appears that strain 19 had about the same agglutinogenic power as strain 544. The route of inoculation had a definite effect on the agglutination titres, those in the intravenous and intracerebral groups being significantly higher than those in the intramuscular and intraperitoneal groups (chi squared=19·4 corresponding to a value for P of less than ·001).

In the case of mice inoculated with the small dose (300 viable organisms) agglutinins could only be demonstrated in the sera in four instances.

Histological findings.—Brains, livers and spleens from two infected mice were removed at one and five weeks after inoculation and examined histologically. The following changes were observed. The brain showed an encephalitis with numerous foci of mononuclear cells, often in close proximity to a blood vessel. In one area a gliosis was suspected but special staining was not applied to confirm this. A lesion was present in the cortex superior to the hippocampus, and here a slight cellular infiltration containing a few polymorphic cells surrounded an area of roughly 1 sq. mm. which contained chromatin dust and degenerated nuclei. The cerebellum and choroid plexus appeared normal but the meninges, where visible, showed an infiltration with mononuclear cells. The liver was in a state of cloudy swelling with some nuclei showing karyorrhexis. Loss of the columnar arrangement of the parenchyma was also observed as well as an infiltration with mononuclear cells of Glisson's capsule and of the sinusoids. A few of these cells showed mitotic figures and some were arranged in the sinusoids in small foci resembling giant cells ; two venules were in a condition of stasis. The spleen showed congestion with areas of oedema.

SUMMARY AND CONCLUSIONS

(1) Mice were inoculated with *Brucella abortus*, strains 544 and 19, and the progress of infection studied. The infection was of a chronic type which in no instance caused the death of the infected animal.

(2) The strain more virulent for cattle and guinea-pigs (strain 544) propagated in the mouse rather more readily than did strain 19. The difference in behaviour of the two strains was much less marked than it is in the guinea-pig.

(3) The route by which the organisms were introduced had a very definite effect on their subsequent propagation. Intravenous or intracerebral inoculation resulted in a far higher degree of infection than did intramuscular or intraperitoneal inoculation.

(4) It was shown that the infection attains its maximum extent within a week from inoculation. The extent of infection had not significantly changed at three and five weeks after inoculation.

(5) The rate of propagation of the parasite was strongly influenced by the size of the original inoculum. With as few as 300 organisms only a very slight infection was produced which showed no signs of progressive development.

(6) Intracerebral inoculation of mice with *Br. abortus* resulted in a rapid but rather transient infection of the brain accompanied by a more persistent infection of the liver and spleen.

(7) Agglutinins could be demonstrated in the blood of mice inoculated with the larger dose of organisms (300,000) five weeks after inoculation. The strain of *Brucella* used did not influence the agglutination titre.

(8) Pathological changes in the brains, livers and spleens of infected mice are described.

It can be concluded from the above findings that the mouse does not become readily infected with *Br. abortus*, though infection, once established, tends to persist for a considerable time. For the purpose of comparing the degree of virulence of different strains of *Br. abortus*, the mouse is much less valuable than the guinea-pig.

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REFERENCES

Ber, A. (1933). *Zschr. f. Infektkr.*, **44,** 129.
De Ropp. (1945). *J. comp. Path.* **55.** 70.
Fabyan, M. (1912). *J. med. Res.*, **26,** 441 : (1913). *Ibid.*, **28,** 81.
Feldman, W. H., and Olsen, C. (1935). *J. infect. Dis.*, **57,** 212.
Hagan, W. A. (1922). *J. exp. Med.*, **36,** 727.
Holth, H. (1911). *Zschr. f. Infektkr.*, **10,** 206 and 342.
Kolmer, J. A., Bondi, A., and Rule, A. M. (1940). *J. infect. Dis.*, **67,** 268.
Priestley, F. W., and McEwen, A. D. (1938). *J. comp. Path.*, **51,** 282.
Scorgie, N. J. (1938). *J. Path. Bact.*, **46,** 165.
Singer, C., and Shaw, E. B. (1935). *J. Bact.*, **29,** 44.
Singer-Brooks, C. H. (1937). *J. infect. Dis.*, **60,** 265.

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